University of Akureyri
Faculty of Business and Science
Department of Natural Resource Sciences

Thermophilic ethanol and hydrogen production from lignocellulosic biomass

Arnheiður Rán Almarsdóttir

Supervisor: Dr. Jóhann Örlygsson
University of Akureyri
Department of Business and Science
Faculty of Natural Resource Science
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"Every scientific truth goes through three states: first, people say it conflicts with the Bible; next, they say it has been discovered before; lastly, they say they always believed it."

~Louis Agassiz~

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Declaration

I hereby declare that I am the only author of this thesis and it is the product of my own research

Arnheiður Rán Almarsdóttir

It is hereby confirmed that this master thesis is satisfactory to M.Sc. – degree from the Faculty of Business and Science, department of Natural Resource Science.

Dr. Jóhann Örlygsson

Abstract

Two anaerobic, thermophilic bacteria were isolated from a hot springs in 2005 one from the Krafla area (Víti) in NE Iceland (AK_{17}, belongs to *Thermoanaerobacterium*) and the other from Grensdalur in SW-Iceland (AK_{14}, belongs to *Clostridium*).

For AK_{14} the focus was on hydrogen production. Growth experiments were performed to investigate growth characteristics, degradation and the formation of fermentation end-products from glucose and xylose. Effect of different glucose concentrations were investigated, substrate spectrum and tolerance to partial pressure of hydrogen. Additionally, hydrolysates from Whatman paper and various complex biomass (grass, straw, hemp and news paper) were pretreated with heat, chemicals and enzymes. The strain was inoculated into anaerobic medium containing 5.0 g L^{-1} of hydrolysates. Fermentation of end products was measured. The liquid/gas ratio clearly affects the hydrogen capacity of the strain as well as other end product produced. The strains produced most hydrogen on cellulose hydrolysate or 8.5 mol H_2 g^{-1}, but less on news paper and lignocellulosic biomass (between 0.26 to 3.60 mol H_2 g^{-1}). When acid or base was used for pretreatment highest production of hydrogen was from grass or 6.23 mol H_2 g^{-1}.

For AK_{17} the focus was on bioethanol production. Effect of environmental factors were investigated, growth on sugars and lingocellulosic biomass. Basic experiment has been performed before. Effect of substrate concentration and substrate utilization tested. Ethanol yields on glucose and xyulose were 1.5 and 1.1 mol/mol sugars, respectively. Experiment with hydrolysates was done, effect on different concentration of hydrolysates, enzymes and acid/base on end product formation. And in the end all optimal condition was used and optimization experiment was done, resulted in 5.5 and 8.6 mM g^{-1} ethanol from grass and cellulose. Additionally effect of inhibitory compounds were investigated, end product formation was clearly inhibited by addition of furfural and hydroxydmethylfurfural. It revealed a total inhibition in end production formation from glucose at 4 and 6 g^{-l}, respectively.

Pretreatment is very important tool when lignocellulosic biomass is used. Then more of cellulose and hemicellulose is free in the hydrolysates which the bacteria can then ferment and produce, hydrogen, ethanol, and acetate.

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

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I would like to thank Dr. Jóhann Örlygsson for giving me the opportunity to work on this project, his guidance and support. I would also like to thank my coworkers, Beata Wawiernia, Alicja Tarazewicz and Margrét Auður Sigurbjörnsdóttir for all their help during my lab work. Furthermore I would like to thank my friends, Hildur Vésteinsdóttur and Sigríður Jóhannesdóttur for their patience and moral support, without them this would never have been possible, my family Elín Steingrímsdóttir and Þorgeir Gunnarsson for believing in me and taking all the calls when I was stressed out. And specially thanks to my grandma, Þorgerður Egilsdóttir, for being my strongest believer even though she never could remember the name of the education I was studying.

Special thanks to Hildur Vésteinsdóttir and Sean M. Scully for proofreading.

Thank you all

Arnheiður Rán Almarsdóttir

Útdráttur

Tvær loftfirrtar, hitakærar bakteríur voru einangraðar úr heitum hverum árið 2007, önnur af Kröflusvæðinu (Víti) á NA Íslandi (AK_{17}) og hin í Grensdal á SV Íslandi (AK_{14}).

Stofn AK_{14} tilheyrir ættkvísl *Clostridium* var sérstaklega rannsökuð m.t.t. vetnisframleiðslu. Framleiðsla á myndefnum við gerjun á glúkósa og xýlósa var könnuð sem og áhrif mismunandi upphafsstyrks af glúkósa á gerjunarferlið. Einnig var mikilvægi hlutþrýstings vetnis athugaður á myndun lokaafurða úr glúkósa. Hlutþrýstingur vetnis hefur greinilega áhrif á vetnis framleiðslu stofnsins sem og á framleiðslu annarra lokaafurða. Hár hlutþrýstingur vetnis minnkar ediskýru, smjörsýru og vetnisframleiðslu en etanólframleiðsla eykst. Útbúin voru „hýdrólýsöt“ úr Whatman pappír og flóknum lífmassa (grasi, hálmi, hampi og dagblaða pappír). Hráefnið fékk bæði hita- og efnameðhöndlun (sýra/basi) auk ensímmeðhöndlunar. Hýdrólýsötin voru síðan sett út í loftfirrt æti (5.0 g L^{-1}) og stofninum sáð í það. Ræktað var í eina viku og lokaafurðir (vetni, etanól, fitusýrur) mældar. Stofnin framleiðir mest vetni á sellulósa hýdrólýsati eða 8.5 mól H_2 g^{-1}, en minna á dagblaða pappír og lignósellulósa lífmassa (milli 0.26 to 3.60 mól H_2 g^{-1}) Þegar notast var við sýru eða basa við formeðhöndlun voru bestu heimturnar af grasi eða 6.23 mól H_2 g^{-1}.

Hjá stofni AK_{17} var megináherslan á etanól framleiðslu. Etanólheimtur úr glúkósa og xýlósa voru 1.5 og 1.1 mól/mól. Áhrif umhverfisþátta á etanólheimtur úr flóknum lífmassa var rannsökuð. Rannsakaða var hvaða áhrif mismunandi styrkur af hýdrólýstötum (Whatman pappír og gras) höfðu á etanólheimtur, sem og mismunandi styrkur af ensímum of efnum (sýru/basa) sem notaðar voru í formeðhöndlun. Að lokum voru niðurstöður notaðar til að rækta stofninn við kjöraðstæður. Þetta leiddi til þess að etanólheimtur voru , 5.5 og 8.6 mM g^{-1} á grasi og sellulósa. Að auki var skoðuð áhrif hindrandi efna, en myndun lokafurða verðu augljóslega fyrir hindrun þegar furfural og hydroxymethylfurfural er bætt í ætið. Það koma í ljós að framleiðsla lokaafurða stöðvast algjörlega við 4 og 6 g^{-1}.

Nauðsynlegt er að notast við formeðhöndlun þegar flókin lífmassi er notaður. Í báðum greinum voru meiri heimtur af lokaafurðum þegar sýra/basi var notaður í formeðhöndlunni. Meira af sellulósa og hemisellulosa er þá laus í hydrolýsatinu, sem bakterían getur nýtt sér og gerjað og framleitt vetni, etanól og ediksýru.

Lykilorð: Hitakærar loftfirrtar bakeríur, vetni, lífetanól, flókinn lífmassi, formeðhöndlun.

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1 Research objective

The objective of this research was to study two thermophilic bacteria for hydrogen and ethanol production from various types of sugars and complex lignocellulosic biomass. Strain AK_{14} is a moderate thermophilic bacterium belonging to the genus *Clostridium*. The bacterium performs the classical acetate-butyrate fermentation pattern with high hydrogen yields. Various physiological experiments e.g. the effect of partial pressure of hydrogen and initial substrate concentrations as well as growth on various carbon substrates, both sugars as well as hydrolysates from lignocellulosic biomass were investigated in detail for the strain. This work has been published in *Icelandic Agricultural Sciences* and is presented in this thesis as Manuscript I (Chapter 5) with the allowance of the editorial board of the journal.

The other strain studied is *Thermoanaerobacterium*strain AK_{17}.This strain has been intensively investigated in recent years because of its ethanol production capacity on carbohydrates. The present investigation studies the effects of various environmental factors (different concentrations of hydrolysates, enzymes and acid/base) on ethanol production capacity using simple sugars and various lignocellulosic biomass. Results are presented in Manuscript II (Chapter 6).

2 Introduction

Energy plays a significant role in our life. Most energy demand is met by fossil fuels (oil, coal and natural gas). Petroleum products are the main transportation fuel and coal is mostly used for producing electricity whereas natural gas is increasingly used for heating (Environmental Literacy Council, 2008). Use of fossil fuels has increased along with improvement of standard of living, the proliferation of the world´s population and the industrialization of the developed countries. It has been recognized for some time that current use of fossil fuels will not only deplete the world´s oil reservoir but also have serious impact on the environment, leading to increased health risk and global climate change (Panwar *et al.,* 2010). It has been estimated that fossil fuels will be depleted by the year 2100 which makes the need for alternative fuels solutions significant (Saxena *et al.,* 2007). Global warming can mainly attributed to an increase in CO_2 emissions which have increased by 30% in the past 200 years. Since 1800 20 Gt of CO_2 has been released to the atmosphere (Panwar *et al.,* 2010).

Renewable energy, including hydroelectric power, solar, wind, geothermal energy and biomass, causes little or no pollution (European Renewable Energy Council, 2008). These renewable energy sources have great potential to meet energy needs of the future. Fuel produced by the activity of microorganisms, such as ethanol, methane and hydrogen, are called biofuels (Drapcho *et al.,* 2008). The most common biofuels are biodiesel, bio-ethanol and biogas. More than 50% of bio-ethanol produced today is from corn and more than one-third from sugarcane. USA and Brazil produce 90% of the bio-ethanol produced in the world, which counts for about 34 and 24,5 billion m^3 , respectively, in 2009 (REN21, 2010; Crago *et al.,* 2010).

2.1 Renewable energy

Renewable energy is classified as energy which regenerates within a short time. There are several types of energy that can be classified as renewable energy: geothermal, solar, wind- and tidal, hydroelectric and energy from biomass. Renewable energy generates less greenhouse gas emissions than fossil fuels and is otherwise pollution-less (Environmental Literacy Council, 2008a). The majority of energy used today is derived from coal, oil and gas. This energy is non-renewable because once used it does not regenerate as it takes millions of years for these chemicals to form and it is estimated that they will be largely exhausted within 60-100 years, depending on which fossil fuel it is (Saxena *et al*., 2007). Clearly, it is necessary to reduce the use of fossil fuels and direct combustion of biomass because of high greenhouse gas emissions. The main obstacle for the utilization of renewable biomass fuels are high production costs. This cost is however declining, but the technology used is still considerably more expensive compared to the technologies used to produce fossil fuels. The capital cost is high for renewable energy production, due to high start-up cost, but the actual implementation costs are less than for fossil fuels in energy output per unit (Environmental Literacy Council, 2008c). Many governments have considered partially subsidizing the capital cost, which would increase the chances of energy production from renewable sources (Environmental Literacy Council, 2008b).

Only 14% of energy used in the world today is derived from renewable energy (Figure 1): the rest of the energy sources are fossil fuels. The rate of renewable energy is expected to rise significantly in the next 90 years, and in 2100 it is estimated that renewable energy will be 30-80% of all energy used in the world (Panwar *et al*., 2010).

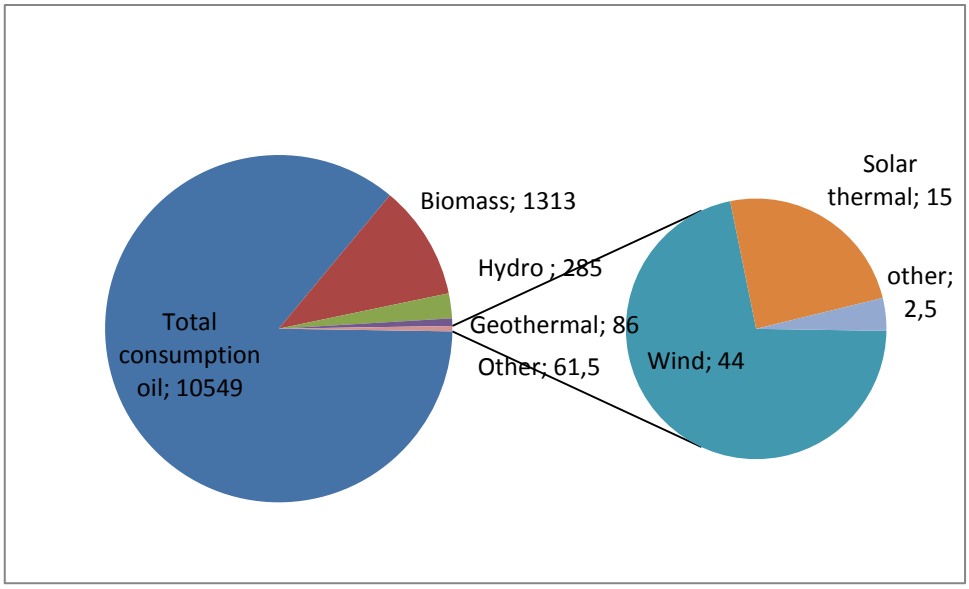

Figure 1- World-wide oil and renewable energy consumption in the year 2010 in million tons. (Panwar *et al.*, 2010)

2.1.1 Geothermal energy

Geothermal energy is energy gained by using the heat from the earth´s hydrothermal systems. In geothermal areas water sinks below the earth surface and warms up. The water is used either as hot water or as steam to drive turbines that produce electricity (Environmental Literacy Council, 2008c). Geothermal heat is considered to be clean and renewable energy although there are some arguments about this issue, some scientists claim that geothermal energy is not completely renewable (U.S Department of Energy, 2005; Palmason, 2005). The range of sources that can be tapped for energy can be tapped from deep surface to shallow ground (U.S Department of Energy, 2005).

2.1.2 Hydropower (hydroelectric power)

Hydropower is produced by using movements of the water to power engines to make electricity. Hydropower has many benefits; high availability, no CO_2 emissions, and because of the simple technologies used to harness hydropower, it has a long history of usage (U.S. Department of Energy, 2005a; Environmental Literacy Council, 2008d). People have utilized water for thousands of years. The Greeks used water wheels 2000 years ago to grind wheat into flour. The technology of using falling water to make hydroelectricity has existed for centuries (U.S. Department of Energy, 2005a). Water is

continuously running through a water cycle driven by the sun which results in energy production. Since hydropower is dependent on water constantly running it is regarded as renewable energy (Environmental Literacy Council, 2008d). To change the water energy into electricity, turbines and generators are used. The electricity goes to an electricity grid, wherefrom it is distributed to end users e.g. homes (U.S Department of Energy, 2005b).

There are still some obstacles producing hydropower, e.g. high capital cost regarding buildings and the water lagoons require huge space and may have negative impact on the environment. But when installed, it has relatively low operational cost and is a very clean energy source (Environmental Literacy Council, 2008d).

2.1.3 Solar power

Energy obtained from the sun is the most abundant and cleanest renewable source available (Panwar *et al*, 2010; Solar Energy Industries Association, 2005). The sun has been burning and emitting heat and energy for more than four billion years (Alternative Energy). There are several technologies used to catch energy from the sun to produce electricity, light, hot water, heat and even cooling (NREL, 2009). Passive solar heating is a simple method utilizing special building materials, design and sitting. The sun´s position is used to gain light and heat. Solar power systems used today, however meet only a small fraction of energy produced in the world. Energy is collected from the sun by solar panels, surrounded by water and air which the sun warms up. Water and air transfer the heat to storage or utilization devices. Several different techniques have been invented and developed to take advantage of solar energy, including photovoltaics (converting light to electricity), concentrated solar power (utility scale) and solar lighting and heating and cooling systems (solar thermal) (Environmental Literacy Council, 2008e; NREL, 2009; Solar Energy Industries Association, 2005). The first solar cell was made 30 years ago but the technique has since then developed fast (Solar Energy Industries Association, 2005). The use of solar energy is difficult to compare to the use of fossil fuels because the solar technology involves a huge initial investment capital and occupies large spaces (Environmental Literacy Council, 2008e).

2.1.4 Wind energy

Wind as an energy source has been used for a long time: first to propel boats but since 1880´s wind power has been used to make electricity (Environmental Literacy Council,

2008f). Wind energy is another type of solar power, since wind is created when the sun shines and heats up the atmosphere creating a temperature gradient. Wind is also caused by the rotation of the Earth and its irregular surface (Environmental Literacy Council, 2008f). The production of electricity from wind is almost fully developed and fully competitive with other renewable energy sources. Wind is a completely pollution free technology and is used in many places around the world. Energy from wind is converted to electricity or mechanical energy by wind turbines which create power by driving a generator (Panwar *et al.*, 2010). As the wind turns the turbine blades a shaft connected to a generator spins, producing electricity. There are few flaws concerning the use of wind energy, the cost of building and installing the turbines is still higher than for generators used for fossil fuels as well as the instability in energy source. However, wind power has been criticized because of the environmental disruption of wildlife especially birds and also because of noise and visual effects (Environmental Literacy Council, 2008f).

2.1.5 Bioenergy from biomass

Biomass is organic matter which is produced by plants, animals and microorganisms (IUPAC, 1997). Energy from the sun is converted to organic matter by green plants, algae and photosynthetic bacteria (Glazer & Nikaido, 2007). By photosynthesis, plants and micro-organisms convert carbon dioxide into carbohydrates e.g. sugars, starch and cellulose (Australian Institute of Energy, 2009). Biomass can be converted to fuels in the form of liquids, gasses (Environmental Literacy Council, 2008a) or to heat when burned. Then, the carbohydrates in the biomass respond to oxygen and form carbon dioxide and water. In a complete burn the same amount of carbon dioxide is formed as the plant has fixed during its growth (Australian Institute of Energy, 2009). Direct burning has been done for centuries but it is not the most efficient method of biomass utilization because of incomplete combustion, low efficiency and pollution. Therefore it would be more feasible to convert the biomass to other fuel forms which are better to handle and pollute less when used. Examples of such fuels are hydrogen, methane, methanol, butanol and ethanol. Biomass fuels are still considerably more expensive than fossil fuels but emerging technologies will decrease this cost in coming years (Ni *et al.*, 2006; Balat, 2010).

2.2. Bioenergy and Biomass

2.2.1 Biofuels

2.2.1.1 Biomethane

Methane is odorless gas composed of one carbon and four hydrogen atoms. Methane occurs naturally as a part of the natural gas coming up from the ground: it is produced microbiologically by methanogens in anaerobic environments like swamps, in garbage dumps and in the digestive systems of many animals. Methane is lighter than air, highly flammable and non-toxic unless presented in large amounts in confined spaces where it may cause suffocation (Ogejo *et al.*, 2007).

Methane is considered to be a greenhouse gas: it has 21 times more greenhouse effect then carbon dioxide (CO_2). Biogas (CH_4 and CO_2) produced in landfills has been collected for many years and used either directly as an energy source (burning) or the methane is separated from CO_2 (and other gases) and used as vehicle fuel. For example, this is done in the landfill in Álfsnes in Reykjavík, Iceland. More commonly, methane is produced by anaerobic digestion from wastewater and agricultural residues, and has been broadly applied both in pilot and large scale facilities, mainly in Denmark and Germany (Metan, 2003; Reith *et al.*, 2001).

Great technological advances have occurred in the production of methane vehicles in recent years. The third generation of methane vehicles uses only methane although it has been more common to convert cars made for petrol and convert them into methane cars (Metan, 2003).

2.2.1.2 Biodiesel

Production of biodiesel is mainly from oil rich plants such as rape oil and soybeans (Hill *et al.*, 2006). Almost all biodiesel production is carried out by catalytic transesterification of oils with a strong base which is cost-effective and does not require high temperatures or pressure. Biodiesel has theoretically 5-8% less energy compared to conventional diesel but because of better lubrication properties the actual energy difference is only 2% lower, or about 35 MJ L^{-1} (Hill *et al.*, 2006).

The production of biodiesel from algae is a potential viable option. Algae species can range from small single-celled organisms (microalgae) to a multi-cell organisms with complex structures. All algae are autotrophic organisms that produce energy through photosynthesis. Microalgae have mainly been used for biodiesel production and often contain high levels of lipids and fatty acids in their cells membranes or as reservoir material. The ratio of lipid/oil by weight of algae varies widely (from 2 to 70%) but is among the highest ratio found in living organisms (Chisti, 2008).

2.3 Biomass

The composition of biomass used for the production of biofuels varies to a great extent. Sugar and starch-rich biomass like corn and sugarcane are examples of easily degradable biomass that, upon hydrolysis, yield mostly glucose and sucrose. Lignocellulosic biomass has a more complex structure and thus requires additional pretreatment in the form of heat, strong acids or bases, or enzymes such as cellulases and hemicellulases (Kosaric *et al*., 2001).

2.3.1 Sugars

The main sugar used for biofuel production is the hexose, glucose (Figure 2), primarily from corn, and the disaccharide sucrose (Figure 2) from sugarcane. Biofuel produced using these sugars are called first generation biofuels, opposite to second generation ethanol from lignocellulosic biomass. Most microorganisms ferment sugars easily via the Embden-Meyerhof pathway to pyruvate and convert it to ethanol by using alcohol dehydrogenases (Lynd *et al.,* 2002). Sugar beet contains about 20% sucrose composed of glucose and fructose that are linked together by α-1.2 glycosidic bond. The yeast *Saccharomyces cerevisiae* has the enzyme invertase, which can hydrolyze sucrose and thus split it into glucose and fructose which can then be fermented to ethanol. Other types of biomass that are rich in sugars are e.g sweet sorghum and many fruits (Glazer & Nikaido, 2007).

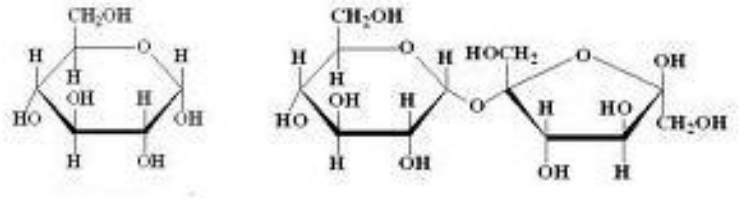

Figure 2 Structure of glucose (to the left) and sucrose (to the right)

2.3.2 Starch

Several species of starch rich plants are suitable for biofuel production, e.g. corn and sweet potatoes (Kosaric *et al*., 2001). Corn starch (Figure 3) is the main raw material for bioethanol production in the USA. It consists of two polymers, the water soluble amylose (20%) and the water insoluble amylopectine (80%) (Burgoyne, 1988). Amylose is a linear polymer of D-glucose units linked together by α-1.4-glucosidic bonds. The number of D-

glucose units in a single chain of amylose ranges from 200 to 500 units. Amylopectin is a branched polymer consisting of D-glucose units with α-1.4-glucose bonds, as well as α-1.6-associated with approximately 25 sugars apart at the branching points of the polymer (McMurry & Simanek, 2007).

Two enzymes are used to hydrolyze α-1.4-bonds in starch, α-amylase and β-amylase. α-amylase breaks down the starch into glucose and maltose, while the β-amylase hydrolyses the α-1.4 bonds which are not accessible for α-amylase. These two enzymes do not completely degrade amylopectine because they cannot hydrolyze the α-1.6-bonds. To break these bonds another enzyme, amylo-1.6-glucosidase, is needed. When these enzymes are used together they can hydrolyse amylose and amylopectine into maltose and glucose. The enzyme maltase is finally needed to degrade maltose to two glucose units (Perry, Staley & Lory, 2002).

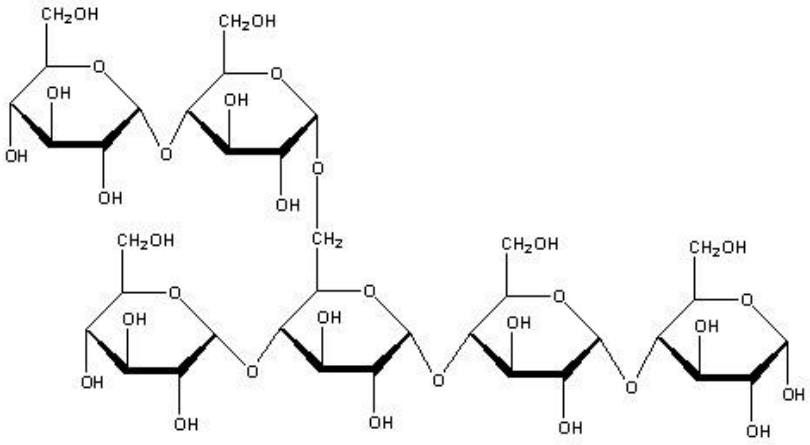

Figure 3 The main structure of starch; polymer of glucose with α-1.4 and α-1.6 links.

2.3.3 Lignocellulosic biomass

The use of biomass for biofuel production which can also be used for human consumption (e.g. sugar beets, sugar cane and corn) is very controversial and has caused a food-versus-fuel debate worldwide since the amount of such biomass is limited. Therefore, the interest in other types of biomass has emerged in recent years. Lignocellulosic biomass is an example of such biomass. It is available in immense amount and is present in all plants (Hahn-Hägerdal *et al*., 2006). Lignocellulose is composed of cellulose, hemicellulose and lignin (Figure 4). These three different polymers bind to each other with hydrogen bonds

with covalent cross-link that forms the complex lignocellulose that consist of more than 90% dry weight of plant cells (Balat, 2010).

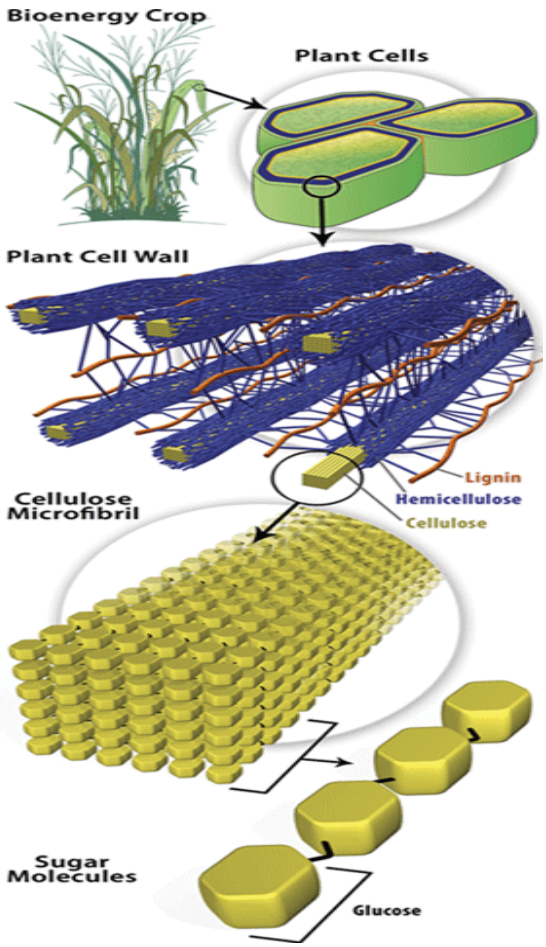

Figure 4 Structure of lignocellulose (Bioenergy, 2008)

The three polymers are tightly bound together and need to be separated from each other before fermentation. Hemicellulose and lignin generate some structural defense around the cellulose that must be removed before cellulose is hydrolyzed (Hamelinck *et al.*, 2004). The amount of hemicellulose in plants is highest in grasses but in trees the percentage of different compounds are 36-61% of cellulose, 13-39% of hemicellulose and 6-29% of lignin (Olson & Hahn- Hägerdal, 1996). The production of lignocellulose on earth is about 2 to 5 $\times 10^{12}$ tons every year (Wyman *et al.*, 2005; Glazer & Nikaido, 2007).

2.3.3.1 Cellulose

Cellulose is the most abundant organic material on earth. Cellulose does not dissolve in water, has a high tensile strength and has much higher tolerance to degradation compared to glucose in starch (Glazer & Nikaido, 2007). In its natural form, cellulose is a linear polymer containing thousands of glucose units liked together by β-1.4-glucose bonds (Figure 5) (McMurry & Simanek, 2007). Two glucose units form cellobiose which is the fundamental unit of the polymer. The polymer has a surface which is ribbed and supported by hydrogen bonds that are located both internally within a single strand as well as between adjacent chains. The chains are long strands of crystallized bonds so called microfiber bonds and are 250 Å wide. Then the chains connect together to form a larger fibers. These fibers form a thin layer that form a variety of building blocks of plant cells. Structure of cellulose can have crystalline or amorphous region, depending on the source and combination (Glazer & Nikaido, 2007). Cellulose is very tolerant towards degradation due to secondary and tertiary structure of the cellulose chain and how it is integrated with other polymers (lignin, starch, pectin, hemicellulose and protein) in the cell wall plant (Kosaric *et al*., 2001).

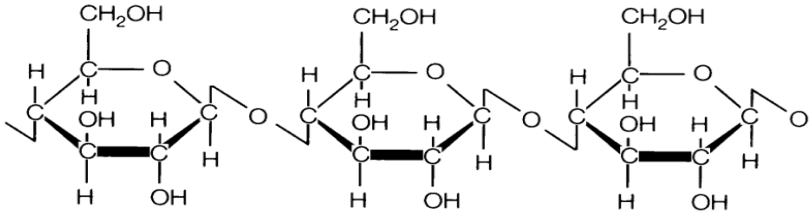

Figure 5 - The main structure of cellulose

2.3.3.2 Hemicellulose

Hemicellulose is a complex polysaccharide, structurally similar to cellulose but its main bonds are 1.4 that connect β-D-pyrosyl units (Figure 6). Hemicellulose connects lignin and cellulose together and gives the lignocellulose structure greater strength (Hendriks & Zeeman, 2008). The polymer is usually shorter than cellulose (< 200 units) and is highly branched with many different kinds of sugars e.g pentoses (xylose and arabinose), hexoses (galactose, mannose and glucose) and sugar acids (D-glucoronic acid) (Glazer & Nikaido, 2007). The primary structural unit in hardwood hemicellulose and agricultural plants is xylan, but in the main building unit of softwood hemicelluloses is glucomannan (Hendriks

& Zeeman, 2008). The polymer is in the cell walls of most land plants and is the primary material in mature wood fibers (Perry, Stanley, & Lory, 2002).

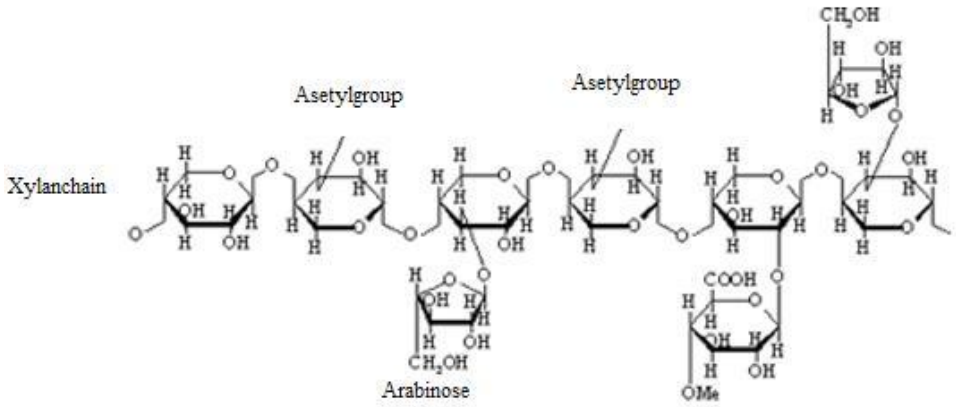

Figure 6 Structure of hemicelluloses

2.3.3.3 Lignin

Lignin (Figure 7) is present in all lignocellulosic biomass and is the most common aromatic polymer on earth. The structure is an amorphous heteropolymer consisting of three different phenyl propane units (p-coumaryl, coniferyl and sinapyl alcohols) which are bound together with different chemical bonds (Hendriks, & Zeeman, 2008).

Twenty to thirty percent of wood fibers are composed of lignin. ”Higher“ plants and ferns contain lignin but ”lower“ plants, like mosses, do not (Perry, Stanley & Lory, 2002). The primary purpose of lignin is to give the plant strength, impermeability, protection against microbial attacks and invasion of oxygen (Hendriks & Zeeman, 2008). Because of its strength, trees are able to grow up to several hundred meters (Glazer & Nikaido, 2007). Resistance to microbial degradation of lignin can be attributed to its production. Lignin is formed from three alcohols (the lignols) with free radical copolymerization (Perry, Stanley & Lory, 2002).

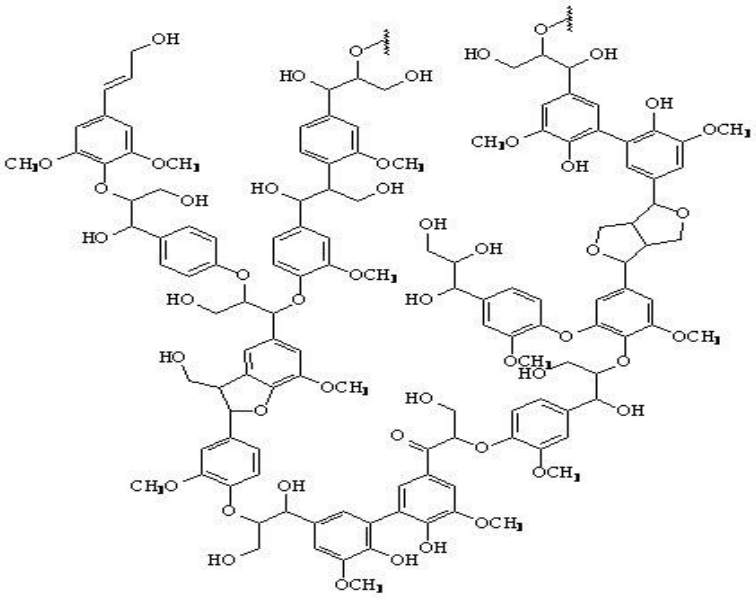

Figure 7 Main structure of lignin

Lignin is classified into softwood lignin, hardwood lignin or grass lignin. Softwood lignin is mainly composed of coniferyl alcohol and small quantities of *p*-coumaryl alcohol. Hardwood lignin is 8% *p*-hydroxyphenylpropane units (derived from *p*-coumaryl), coniferyl, and sinapyl alcohol in equal amount. Grass lignin consists of sinapyl, coniferyl, and *p*-hydroxyphenylpropane units and *p*-coumaryl alcohol side chain use to esterified *p*-coumaric acid (Figure 8), (Glazer & Nikaido, 2007).

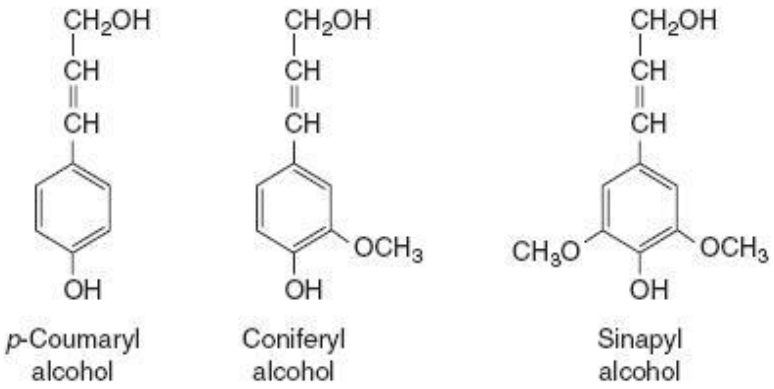

Figure 8 Three main chemicals in lignin

Few organisms in nature are capable of degrading lignin although some species of fungi can degrade it and use its degradation products as carbon source but not as energy (Perry, Stanley & Lory, 2002).

2.3.4. Pretreatments of lignocellulosic biomass

The main goal of the various pretreatment methods known is to separate the three components of lignocellulose: cellulose, hemicellulose and lignin and enhance the accessibility and susceptibility of the cellulose and hemicellulose. This is done by reducing biomass particles size and change the biomass structure to make it more accessible to enzymes. The goal of all pretreatments is to change and remove ingredients that are inhibiting the hydrolysis of ligocellulose so that they can increase the activity of enzymes. These enzymes convert carbohydrate polymers into sugar which can then be fermented (Mosier *et al*, 2005; Srinivasan & Ju, 2010; Hosseini & Shah, 2009; Balat *et al*., 2008).

Several requirements are needed for pretreatment of biomass to make it economically feasible (Balat *et al*., 2008):

1. Good monosaccharide yields
2. Minimize loss and degradation of carbohydrates
3. Minmize the formation of substances that may have inhibitory effects on the hydrolysis and fermentation process
4. Inexpensive

Studies have shown that pretreatment of lignocellulose is the major factor determining the recoveries of sugars from the hydrolysis of cellulose and hemicellulose (Balat *et al*., 2008). Several different types of pretreatment are used today and are described below.

2.3.4.1. Steam explosion

Steam explosion is widely used pretreatment method. The biomass is placed in a closed chamber and treated with hot steam (up to 240°C) and high pressure for 1 to 5 minutes. The biomass is quickly cooled down causing an increase in the volume of the vapor inside the biomass leading to separation of the fibers. The purpose of steam explosion is to dissolve the hemicellulose and make cellulose more accessible for enzyme treatment as well as preventing the formation of inhibitory substances (Hendriks & Zeeman, 2008). It is believed that the hemicellulose is hydrolyzed by acetic acid and other acids produced during steam explosion, but water also acts as acid at high temperatures. Acid catalysts are sometimes used with steam explosion to achieve higher sugar yields. Acids used are preferably H_2SO_4 and SO_2 who increase sugars recovery from the hemicellulose and also improve the functionality of enzymes which then hydrolyze the cellulose. These two acid catalysts give a different response: H_2SO_4 gives high recoveries of sugars from the

hemicellulose but inhibitory substances are often produced whereas the use of SO_2 leads to lower sugar recoveries but fewer amounts of inhibitory substances (Balat *et al*., 2008).

2.3.4.2 Liquid hot water pretreatment

Liquid hot water (LHW) methods include using pressure to keep the water in a liquid phase at elevated temperatures. The biomass is submerged in hot water (200-230°C) for several minutes leading to partial (40-60%) dissolution of the lignocellulose. More than 90% of the sugars present in the hemicellulose fraction are recovered but 4-22% of the cellulose fraction may be lost (Mosier *et al*., 2005).

The main difference between the LHW and steam explosion is that the amount of dissolved product is higher in the LHW compared to the steam explosion exposed biomass, but the concentration of the products is greater with the steam explosion then with LHW. This is caused by the large amount of water used in LHW (Hendriks & Zeeman, 2008).

2.3.4.3 Acid pretreatment

Pretreatment with mild acid is the most studied and is the most common pretreatment used today (Balat *et al*., 2008). Using acid pretreatment usually gives good recoveries of sugars from lignocellulosic biomass. There are several variations of acid pretreatment and several acids (H_2SO_4, HCl, HNO_3, and H_3PO_4) can be used although the use of sulfuric acid is most prevalent. The temperature range used for acid pretreatment is usually from 160 to 220°C and is held for several minutes. The acid concentration can be either strong or weak to remove the hemicellulose and to improve the hydrolysis of the cellulose (Balat *et al*., 2008). The use of strong acids is not favorable in terms of ethanol production because of increased production of inhibitory substances. The use of weak acids is considered to be more efficient, and causes fewer problems in terms of equipment damage (Hendriks & Zeeman 2008; Mosier *et al*., 2005).

Higher temperatures and shorter pretreatment times usually results in better recoveries of xylose and improve the access of enzymes to hydrolyse cellulose (Balat *et al*., 2008).

2.3.4.4 Alkaline pretreatment

Alkaline pretreatment such as NaOH and $Ca(OH)_2$ are used to remove lignin and part of the hemicelluloses in complex biomass. The accessibility of cellulose increases largely as well as glucose from hydrolysis of cellulose (Hamelinck *et al*., 2004). Pretreatment with alkali takes place at lower temperatures, lower pressure and longer time compared to other pretreatment methods (Mosier *et al*., 2005). The use of alkali pretreatment, however, has its disadvantages, since a part of the alkali will cause salt formation that may be harmful to the environment and lead to difficulties to reuse the bases (Hamelinck *et al*., 2004).

2.3.4.5 Biological pretreatment

Biological pretreatment is sometimes used on biomass. Fungi can break down the lignin fraction of the lignocellulose. This method is used at low temperatures and mild conditions (Zhang, 2007a). Most fungi degrading lignin grow at 20-30°C. One fungus, *Phanerochaete chrysosporium*, has higher optimum temperature (40 °C) and can grow up to 50°C. It breaks down lignin very fast, or up to 3g of lignin/g cell per day (Balat *et al*, 2008). Even though this technology could simplify the removal of lignin significantly, the end product formation is not efficient (Balat *et al*., 2008).

2.3.5. Formation of inhibitory compound by pretreatment

As previously stated, the goal of biomass pretreatment is to break down the basic units of the lignocellulose into monosugars. Depending on the pretreatment used, a portion of the sugars and other organic compounds present can be converted to other substances, such as furfural, hydroxymethylfurfural, acetic acid, syringic acid, p-hydrobezen acid, and vanilline. These compounds have and inhibitory effects on growth and metabolism of microorganisms (Mussatto & Roberto, 2003). Therefore, efforts are usually made to minimize the formation of these chemicals or to remove them from the hydrolysates before fermentation (Hendriks & Zeeman, 2008). Furfural and hydroymethyfurfural have similar activity; they retard the fermentation of hemicellulose hydrolysates by yeast or other biocatalysts and must be removed or mitigated. Furfural is considered more toxic (Zaldivar *et al*., 1999). Relatively low concentration of these substances has inhibitory effects on microbes; complete inhibition has been observed for yeast between 1.0 to 2.0 g L^{-1} (de Vrije *et al*., 2009). Other inhibitory compounds formed during pretreatment of biomass

include phenols, acetic- formic- and levulinic acid and aromatic substances formed by decomposition of lignin (Mussatto & Roberto, 2003).

2.3.6 Removing inhibitory compounds from hydrolysates

Fermentation of hydrolysates with and without inhibitors clearly shows that yields are usually lower in untreated hydrolysates (undetoxified). The inhibitors act as strong barriers for microbial metabolism. Consequently, it is important to remove or neutralize these compounds from the hydrolysates before fermentation (Mussatto & Roberto, 2003).

Several detoxification methods (biological-, physical- and chemical) have been used to convert inhibition compounds into inert materials or to reduce their concentration. The effectiveness of a detoxification method is dependent on the type of the hemicellulose in the hydrolysate and the microorganisms used for the fermentation of the hydrolysate. Before a detoxification method is chosen the chemical composition of the hydrolysate must be taken into account, but it changes depending on the materials used and the pretreatment method used (Palmquist & Hahn-Hägerdal, 2000).

Biological methods using special enzymes or microorganisms that degrade inhibitory substances that are present in the hydrolysate and change them. The use of wood hydrolysate treated with lactase and peroxidase enzymes from white-rot fungus, *Trametes versicolor*, have shown that glucose utilization and ethanol production increases (Palmquist & Hahn-Hägerdal, 2000). The use of soft-rot fungus, *Trichoderma reseii,* has also given good results for the same purpose (Palmquist & Hahn-Hägerdal, 2000). Physical processes, such as vacuum evaporation, have been used to remove volatile growth inhibiting substances, such as furfural and vanilline, from HL. The main drawback of this method is that while reducing the concentration of volatile inhibitory substances the concentration of lignin derivatives increases (Mussatto & Roberto, 2003).

Pretreatment methods using alkali and acid to remove inhibitory substances from hydrolysate are classified as chemical methods and were first described in1945 (Palmquist & Hahn-Hägerdal, 2000). This methodology involves raising the pH of the hydrolysate to pH 9-10 with $Ca(OH)_2$ and then reducing it to pH 5.5 with H_2SO_4; this is effective because many inhibitory substances are unstable at high pH and are thus degraded by the initial base treatment (Palmquist & Hahn-Hägerdal, 2000).

2.3.7 Enzyme pretreatment

During the chemical pretreatment of biomass, the major components of lignocellulosic biomass (lignin, cellulose, hemicellulose) are disrupted resulting in a mixture of various carbohydrate polymers. The lignin fraction of lignocellulose cannot be used for biofuel production and to use the cellulose and hemicellulose fractions, these polymers need first to by hydrolysed to mono- or disaccharides before fermentation. Thus, after chemical pretreatment, an enzymatic treatment of the biomass in usually required. This ”extra step“ is one of the bottleneck concerning the use of lignocellulosic biomass for biofuel production because of high costs (Sun & Cheng, 2002). Well successful pretreatment will largely separate the hemicellulose from cellulose for hydrolysis. Enzymatic degradation of cellulose can give virtually non-toxic HL with a higher concentration of sugars (Olafsson *et al.*, 2008).

There are various enzymes available that can be used to degrade cellulose and hemicelluloses (Mielenz, 2001; El-Zawawy *et al*., 2010; Shih & Smith, 2009). The most effective are cellulases that originate from the fungi *Trichoderma reseii* and also from thermophilic bacteria (Sun & Cheng, 2002). Cellulases are classified into three groups; 1) endoglucanase which breaks β-1.4-glycosidebonds within the cellulose chain, 2) exoglucanase violating coupling ends of the cellulose chain and 3) β-glucosidase which degrades cellubiose to glucose (Grey *et al*., 2006). All three enzymes are needed for a complete degradation of cellulose to glucose (Mielenz, 2001). Many enzymes are also available that can break down hemicellulose. These hemicellulases are divided into many subcategories depending on the mode of action and the type of substrates (Collins *et al*., 2004).

2.4 Fermentation of carbohydrates

Organisms gain energy either through respiration, photosynthesis or fermentation. Respiratory organisms use oxygen as the final electron acceptor but fermentation occurs under anaerobic conditions and is more common in prokaryotes than eukaryotes (Perry, Staley, & Lory, 2002). In fermentation, there is no external electron acceptor. Rearrangement (oxidation and reduction reactions) of the organic compounds used as carbon source leads to release of energy from high energy compounds and ATP is formed by substrate level phosphorylation (SLP) from ADP and inorganic phosphate. The amount of energy produced under anaerobic conditions is much less as compared to respiration, e.g. fermentation of glucose to ethanol and lactate only leads to production of 2 ATP‘s compared to maximum of 38 ATP´s from glucose oxidation in respiration (Madigan *et al*., 2003). The main reason is because of the excretion of these compounds out of the cells instead of a complete oxidation to carbon dioxide as in respiration. In respiration, ATP is formed by oxidative phosphorylation (OP), by creating a proton motive force across the cellular membrane (in prokaryotes) and ATP formation via ATP-ases. (Perry, Staley, & Lory, 2002). Most anaerobic bacteria use glycolysis (Embden-Meyerhof pathway) to break down glucose into two units of pyruvate in a series of ten enzymatic reactions. The glycolysis is also the first part of the degradation process of glucose by aerobic bacteria (Figure 9) (Madigan *et al*., 2003; McMurry & Simanek, 2007).

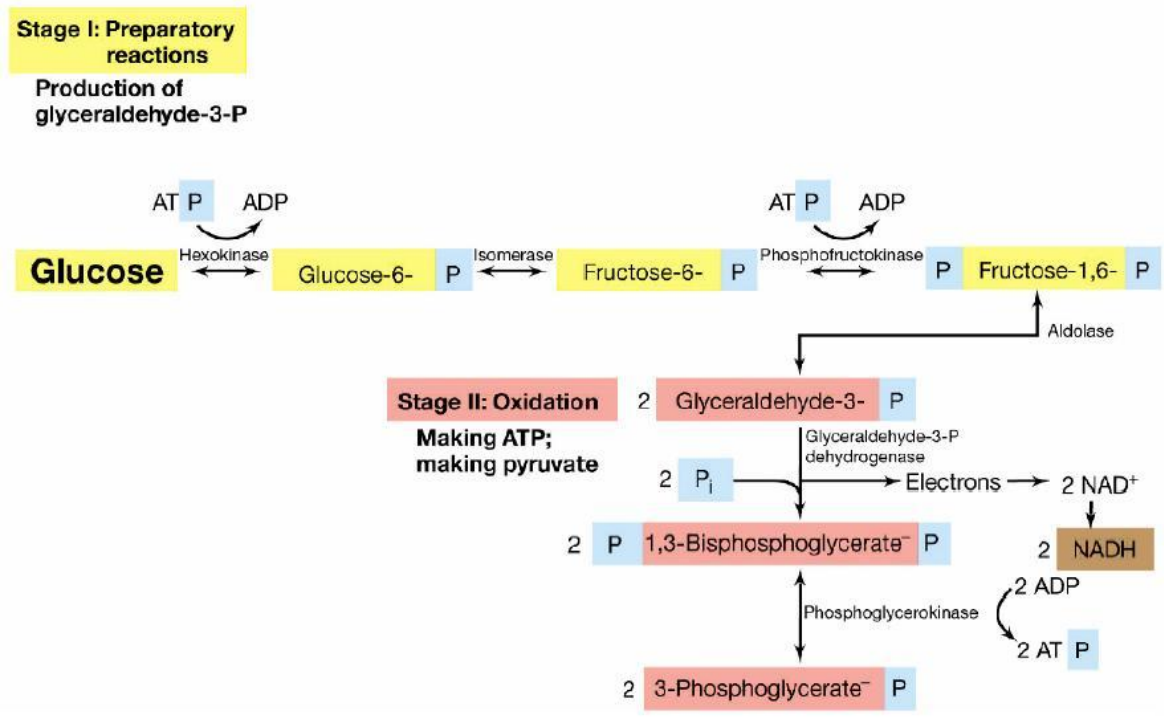

Figure 9 First steps of glycolysis, preparation and oxidation reactions (Madigan *et al*., 2003)

Glycolysis is usually divided into two main parts: preparatory- and oxidation reactions. The end product of glycolysis is pyruvate which can be degraded to various end products. During the preparatory stages of glycolysis, glucose is split up into two molecules of glyceraldehyde-3-phosphate (no oxidation/reduction) in four steps. This "extra" step require two molecules of ATP. In the second stage (oxidation) (Figure 10) two molecules of pyruvate are formed (6 reactions) as well as four ATP and two NADH. The fate of pyruvate is different in depending on the metabolism of the organism, but under anaerobic conditions it is most often reduced to rehydrogenate NAD^+ to NADH (Madigan *et al*., 2003). Thus, pyruvate is converted into various end products such as ethanol, acetic acid, lactic acid, carbon dioxide and hydrogen (Madigan *et al*., 2003).

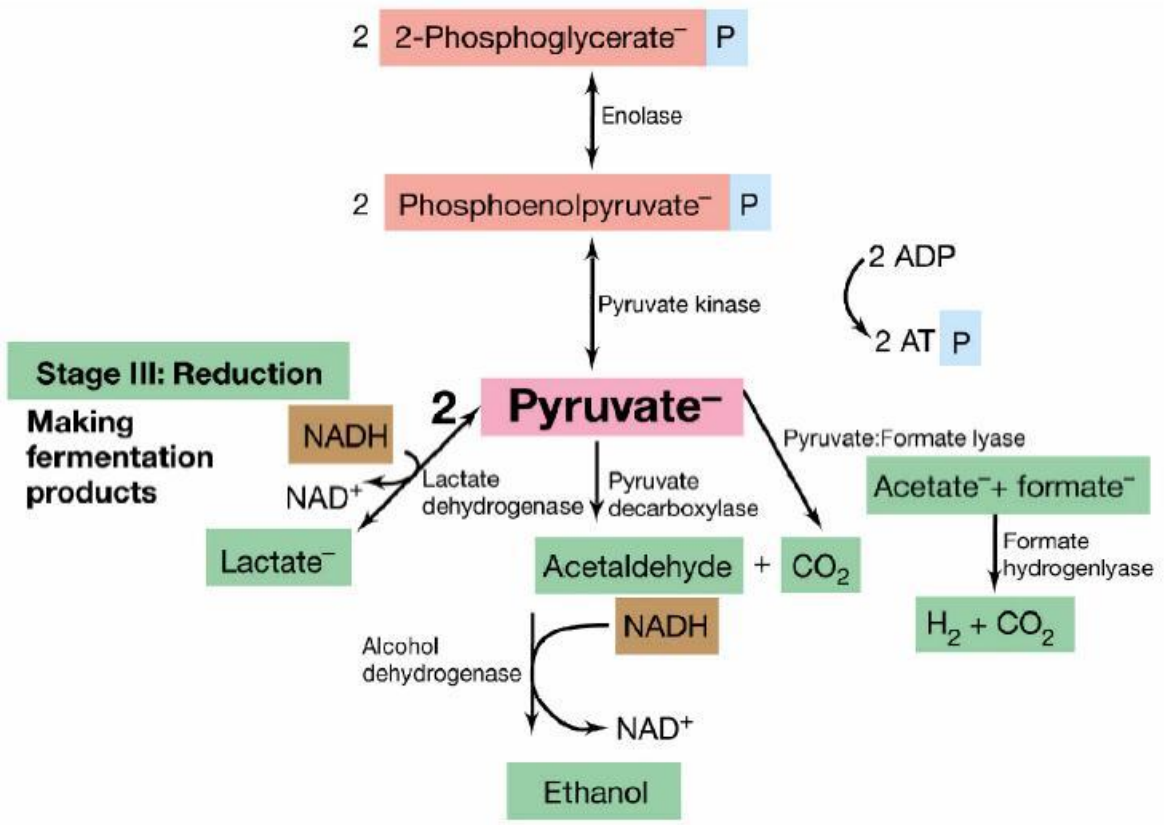

Figure 10 Formation of fermentation products (Madigan *et al*., 2003)

2.4.1 Bioethanol

Ethanol is a colorless, flammable and volatile liquid which boils at 78.4°C and freezes at -114.1°C. Bioethanol is produced from fermentation of organic material by microorganisms. Bioethanol is in many ways the most potential resource as a renewable energy source used today (Mielenz *et al*., 2001). It has been promoted as the main biofuel

in the world and originates mainly from plants such as sugar cane and corn. Ethanol from sugar and starch based biomass has been called first generation ethanol. Cellulosic bioethanol production has recently emerged but its production is much less as compared to 1°-generation ethanol. Ethanol from cellulosic biomass is called second generation ethanol as it is made from sugars derived from cellulose and hemicellulose, which are the main building blocks in complex biomass. Bioethanol from cellulose is considered to be more sustainable fuel than corn and sugar based ethanol in the near future (BioBasics, 2006; Hahn-Hägerdal *et al*., 2007).

Bioethanol has higher octane number (108), burns faster and has higher evaporation temperature than gasoline. These factors results in a higher compression ratio and shorter burn which leads to better energy efficiency compared to gasoline (Balat, *et al*., 2008). Use of bioethanol as an additive in gasoline is steadily increasing. The most common mixture is 10% ethanol and 90% gasoline (E10). Higher concentrations of ethanol e.g. 85% (E85), requires special engines and hybrid cars (American Coalition for Ethanol, 2007). Additionally, the proportion of oxygen in ethanol is higher as compared to gasoline and the blended fuel burns better and smaller amount of carbon monoxide is formed, which is formed mainly by incomplete combustion (Mielenz, 2001; Sánchez & Cardona, 2008; Lynd *et al*., 2002). There are several disadvantages to use bioethanol as a fuel. The energy released by burning ethanol is only 65-69% of the energy released by burning the same amount of gasoline. Also, ethanol has low flame luminosity and low vapor pressure which results in engine ignition difficulties in cold weather. Despite these disadvantages bioethanol is considered an attractive biofuel that is renewable and reduces greenhouse gas emissions ((NO_x, SO_x, CO and CO_2). (Balat, *et al*., 2008).

2.4.1.1 Production of bioethanol

Biofuel was first used in 1894 in France and Germany for transportation. In Brazil, ethanol has been used as fuel since 1925, and its use became widespread around Europe and United Stated in early 1800´s. Interest in biofuel as an alternative fuel for transportation has increased hugely since 1980 (Balat, 2010).

In recent years, increasing attention has been directed to ethanol production by thermophilic bacteria. The high growth temperature offers many advantages over yeasts such as easier recovery of end products, reduced risk of bacterial pollution and broader substrate spectrum. These features are important when it comes to producing ethanol from

complex biomass and when used in the SSF technique (simultaneous saccharification and fermentation) where hydrolysis of biomass and fermentation of sugars takes place simultaneously (Avci & Dönmez, 2006; Lens, *et al.*, 2005).

The production of 1°-generation biofuels in general is mainly limited by the lack of arable land in the world and the fact that the same biomass can also be used as food or feed. For instance, in US alone, more than 80% of the arable land available would only fulfill the need for 50% of the vehicle fleet. Additionally, there is an increased debate worldwide weather to use raw material such as grain for fuel production instead of food because of its huge impact on global food markets and food security (Sawin, 2006; Brennan & Owende, 2009). Therefore, increased interest is now on the use of lignocellulosic biomass that is present in large quantities. This is non-food materials from biomass and waste and is expected to be less harmful on land usage, cost and CO_2 emission reduction (Suurs & Hekkert, 2009). The comparison between 1° and 2°-generation of ethanol is shown in Figure 11.

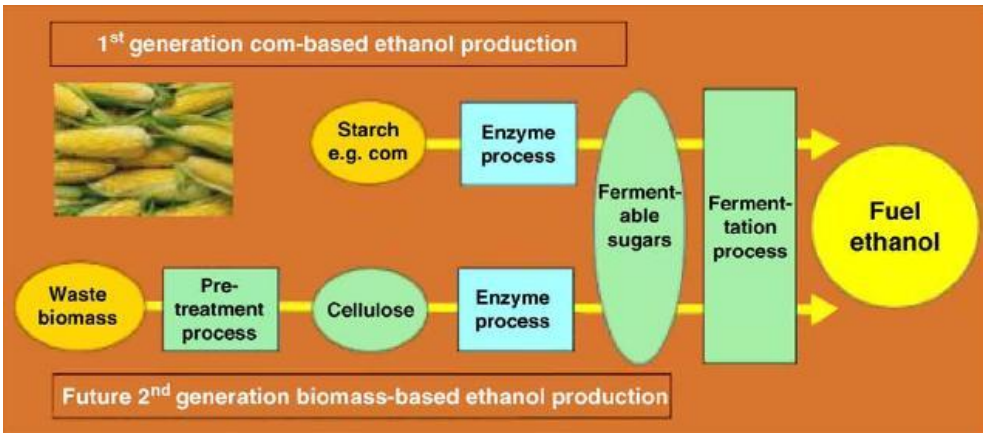

Figure 11 Difference between 1° and 2°generation ethanol production (Martin, 2010)

2.4.1.2 Production of ethanol by fermentation

Ethanol can be produced by many microorganisms from various types of substrates. *Saccharomyces cerevisae* has historically been used mostly for ethanol production from sugar and starch based biomass because of high yields and well known processes. *S. cerevisae* can degrade hexoses and disaccharides present in starch and sugarcane but not pentoses and polymers available in complex biomass. An important aspect of ethanol production by microorganisms from lignocellulosic biomass is recovery of ethanol from fermentation of complex hydrolysates. Another important aspect is the tolerance of

microorganisms to inhibitory compounds formed when biomass is pretreated. Resistance to these agents, including resistance to high concentrations of ethanol, is of great importance for industrial upscaling (van Maris *et al.,* 2006).

No microorganism can meet all conditions that must be met to be considered as a viable option for bioethanol production from complex biomass. The following is a summary of the main characteristics of microorganisms used for ethanol productions must possess:

- Response of ethanol must be greater than 90% of theoretical yields
- Tolerate ethanol concentration exceeding 40 gL^{-1}
- Can grow using simple and inexpensive conditions
- Can grow in the presence of inhibitory substances in undiluted HL
- Can grow at high acid/temperature to prevent pollution in the cultivation (Dien *et al.,* 2003).

2.4.1.3 Production of ethanol by Saccharomyces cerevisiae and Zymomonas mobilis

The yeast *Saccharomyces cerevisiae* has been used for baking and brewing for thousands of years. This yeast is also very important for research in modern cell and molecular biology and is one of the most studied organisms in the world (Kurtzman & Fell, 2005).
Almost all ethanol in the world is produced by yeasts, mostly by *S. cerevisiae*. Yeasts have many good ethanol production qualities but also some disadvantages, e.g. they can only ferment few carbohydrates. The wild type *S. cerevisiae* can for example only ferment monosugars like glucose, fructose, galactose, maltose and sucrose. Although many attempts have been made to engineer the strain, mostly by inserting arabinose and xylose degradation genes, stable cultures are difficult to obtain (Wisselink *et al*., 2008). *Saccharomyces* ferments glucose into ethanol and carbon dioxide by using the EMP with high yields of ethanol. The Gay-Lussac equation (Figure 12) shows theoretical yields of ethanol for glucose degradation. These yields are though never obtained because the yeast needs some of the substrate for growth. The final ethanol yields are in general in the range of 90-95% (Glazer & Nikaido, 2007).

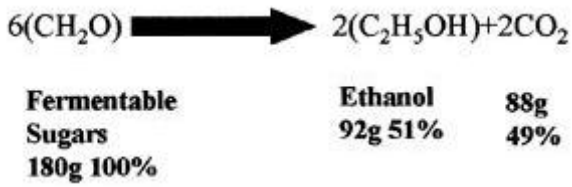

Figure 12 Ethanol yields from sugars with Gay-Lussac equation

Another microorganism which has been widely investigated and is well known for its ethanol production capacity is the mesophilic, Gram negative bacterium *Zymomonas mobilis*. The bacterium can produces ethanol from glucose, fructose and sucrose via Entner-Doudoroff pathway under anaerobic conditions. *Z. mobilis* has unique characteristics, such as high ethanol tolerance, high sugar uptake and high specific ethanol production (Yang *et al.*, 2009; Vasan *et al.*, 2010; Lau *et al*., 2010; Miyamoto 1997). The bacterium has similar ethanol yields as compared to yeasts or slightly higher because they gain only 1 ATP for every glucose degraded and thus a lower portion of the substrate ends in biomass. The bacterium has however been of limited use for ethanol production because it can only degrade glucose, fructose and sucrose (Vasan *et al.*, 2010).

In the past three decades, extensive experiments have been done by scientists to genetically engineer microorganisms to increase ethanol yields and widen their substrate spectrum. Most experiments have been towards cloning in the genes responsible for the degradation of xylose which is the main sugar in hemicellulose fraction of biomass. The most ethanol-efficient experiments have been on genetic engineering on Gram-negative bacteria like *Escherichia coli, Klebsiella oxytoca* and *Zymomonas mobilis* (Dien *et al.*, 2003). In the case of *E. coli* and *K. oxytoca* investigations have mainly focused upon increasing the production of ethanol and reduction of other fermentations products, but both species degrade considerable numbers of sugars (Maris *et al.*, 2002). *Z. mobilis* is however monoethanolgenic and work on this species has thus more been directed to insert genes needed for xylose and arabinose degradation (Yanase, *et al*., 2005).

The main disadvantage of both *S. cerevisae*and *Z. mobilis* is their narrow substrate spectrum. Therefore, an increased interest has been on the use of thermophilic bacteria for biofuel production (Wisselink *et al*., 2008; Yang *et al*., 2009).

2.4.1.4 Thermophilic ethanol producing microorganisms

Thermophilic bacteria capable of producing ethanol were first investigated in the early 1980‘s. The main reason for this interest was because of the oil crisis and the potential of using lignocellulosic biomass instead of sugar and starch (Nardon & Aten, 2008). Several reports were published on *Thermoanaerobacter thermohydrosulfuricus* (former *Clostridium thermohydrosulfuricum*) and *Thermoanaerobacter ethanolicus* showing very high yields of ethanol (Avic *et al*., 2006; Fardeau *et al*., 1996; He *et al*., 2009; Lovitt *et al*., 1988). These bacteria have very broad substrate spectrum, including pentoses and hexoses found in lignocellulosic biomass (Georgieva & Ahring, 2007). The main end-products during fermentation are ethanol but other end products like acetate, lactate and hydrogen reduce ethanol yields (Madigan *et al*., 2003). It is however not until very recently that these bacteria have gained increased attention again. The main reason for increased attention is not only because of expensive gasoline, but because of environmental reasons like greenhouse gas emissions (Singh *et al.*, 2009).

2.4.2.1Ethanol production from sugars

Several bacteria are known to ferment most types of sugars derived from lignocellulose into ethanol some with high yields. These bacteria can, however, have certain disadvantages such as low-alcohol tolerance and the yields are lower as compared to yeasts and *Z. mobilis* because of the production of other end products. Also, there is a substantial lack of knowledge and experience with these bacteria, especially in the industrial scale (Reith *et al.*, 2001).

The best known thermophilic bacteria that produce ethanol belong to the genera of *Clostridium, Thermoanaerobacter,* and *Thermoanaerobacterium* (Koskinen *et al*., 2008a, b, Classen *et al.*, 1999; Lin & Tanaka, 2006; Liu *et al*., 1996; Larsen *et al.*, 1997). Highest yields have been reported from *Thermoanaerobacter ethanolicus*, or 1.9 mol-EtOH/mol glucose (Wiegel & Ljungdahl, 1981).

2.4.2.2 Ethanol production from complex biomass

In recent years more focus has been on using lignocellulosic biomass for ethanol production. Biomass used for the production can be wood and wood waste, energy crops, aquatic plants, agricultural crops and animal waste (Akhtar & Amin, 2010; Alzate & Toro,

2006; Ni *et al*., 2004). It would be possible to produce 442 billion liters every year of bioethanol from lignocellulosic materials by using all complex biomass available in the world (Balat, 2010). The amount of rice straw waste could alone be responsible for 205 billion liters per year, but few investigations have been done one this biomass (Balat, 2010). Several thermophilic bacteria have been reported to give good yield of ethanol, e.g. *C.thermocellum* grown on paddy straw can produce 6.1-8.0 mM/g ethanol (Rani *et al*., 1997), and *Thermoanaerobacter* BG1L1 grown on wheat straw can produce 8.5-9.2 mM/g of ethanol (Georgieva *et al*., 2008).

2.5 Hydrogen

2.5.1 BioHydrogen

Hydrogen gas produced from biomass can be considered as renewable energy carrier. During its combustion, unlike carbon fuels, no carbon dioxide is released, only water vapor. Additionally, hydrogen has a higher energy content (120 MJ/kj) compared to the same amount of other renewable energy sources (Scitopics, 2008; Bromberg *et al*., 1999).

Hydrogen gas can be produced using several methods. Today, hydrogen is by far mostly derived from natural gases, mainly from methane. The gas is mixed together with chemical catalysts and steam at high temperature and pressure to form hydrogen and carbon dioxide (Risø Energy Report 3, 2004). Hydrogen can also be produced by electrolysis when water is split into its elements, hydrogen and oxygen, this is because of it is extremely energy intensive. This method is not widely used and accounts for only 0.1% of the total hydrogen production in the world (Risø Energy Report 3, 2004).

Some microbes can produce hydrogen by either fermentation or photosynthesis in an environmentally friendly way. Such production is called biohydrogen production (Scitopics, 2008).

2.5.2 Hydrogen production

Hereafter, a short description of various ways of hydrogen production is described.

2.5.2.1 Direct biophotolysis

Direct biophotolysis is a biological process where hydrogen is produced from water with solar energy by the following reaction: (Levin *et al.,* 2004; Hallenbeck & Benemann, 2002).

$$\mathbf{2H_2O + light\ energy \rightarrow 2H_2 + O_2} \quad \mathbf{(1)}$$

There are two photosynthesis process systems, Photosystem I (PSI), which produces reductant for CO_2 reduction, and Photosystem II (PSII), which splits water and evolves oxygen. In the PSI process the yields from two photons of water is either reduced CO_2 or hydrogen when hydrogenase is present (Ni *et al.,* 2006).

Under anaerobic conditions green algae and Cyanobacteria (blue – green algae) can form hydrogen by CO_2 – fixation or evolve H_2 as an electron donor. Green-algae contain hydrogenases which are able to produce hydrogen. The enzymes (reversible hydrogenase) are activated and are involved in H_2 metabolisms. It produces protons (H^+) in the medium which forms bonds with electrons and hydrogen (Levin *et al.*, 2004).

For a successful hydrogen production it is critical to keep the oxygen at low level, preferably under 0.1% because hydrogenase is oxygen sensitive. The green-algae *Chlamydomonas reinhardtii* can maintain these conditions by depleting oxygen during oxidative respiration (Ni *et al.*, 2006). Under anaerobic conditions *Chlamydomonas reinhardtii* produced 7.95 mmol H_2^{-1} L^{-1} after 100 hour cultivation (Manish & Banerjee, 2008).

2.5.2.2 Indirect biophotolysis

Indirect photolysis is a process forming H_2 through photosynthesis which is one of the features of *Cyanobacteria*. It is very efficient separating process where O_2 and H_2 evolution phases (the reactions below) are present. Stored carbohydrates are oxidized and H_2 is produced by following general reactions (Ni *et al*., 2006; Levin *et al*., 2004; Dasgupta *et al*., 2010):

$$\mathbf{12H_2O + 6CO_2 + Light\ energy \rightarrow C_6H_{12}O_6 + 6O_2}\ \mathbf{(2)}$$

$$\mathbf{C_6H_{12}O_6 + 12H_2O + Light\ energy \rightarrow 12H_2 + 6CO_2}\ \mathbf{(3)}$$

Thus, indirect biophotolysis involves two different stages coupled through CO_2 fixation where hydrogen and oxygen are produced (Manish & Banerjee, 2008). Enzymes in Cyanobacteria are involved directly in the hydrogen metabolism and synthesis of molecular H_2 (Levin *et al.*, 2004). The hereocystous cyanobacteria has been studied for years and the Cyanobacteria *Anabaena variabilis* has been reported to have the highest yields of H_2 observed, it produces H_2 on spatially separated heterocysts by nitrogenase activity. This strain has demonstrated hydrogen production of 0.355 mmol/H_2 per liter (Manish & Banerjee, 2008; Levin *et al*., 2004; Kotay & Das, 2008).

2.5.2.3 Photo fermentation

Photo fermentation uses energy from light to convert residual organic acids to hydrogen (Ochs *et al.,* 2010). Photo-fermentation is one of the main sunlight-dependent hydrogen production method known (Mathews & Wand, 2009). Purple non-sulfur bacteria use nitrogenase to produce molecular H_2 under nitrogen deficient conditions. For this production organic acids and light energy are needed, as following reaction shows (Levin *et al*., 2004):

$$\mathbf{C_6H_{12}O_6 + 12H_2O \rightarrow 12H_2 + 6CO_2} \textbf{ (4)}$$

Several investigations have been done recently to produce hydrogen from organic waste compounds. Hydrogen can be produced from many different types of biomass waste, such as lactate feedstock and wastewater. *Rhodopseudomonas capsulata, Rhodobacter spheroides* and *Rholdovulum sulfidophilum* have mostly been cultivated on such biomass to produce hydrogen (Levin *et al*., 2004; Hallenbeck & Benemann, 2002; Ni *et al*., 2006). However these bacteria cannot split water themselves because they do not have the mechanisms or the ability. Under anaerobic conditions they can use acetic acid as an electron donor. By using energy in the form of ATP, the electrons can be transported to the nitrogenase by ferredoxin. Hydrogen is formed from protons which nitrogenase reduces and again uses energy in the form of ATP (Manish & Banerjee, 2008).

2.5.2.4 Fermentation

Biohydrogen production from biomass has attracted more and more attention recently (Cheng *et al*., 2010). Using dark fermentation for producing biohydrogen from various organic feedstocks such as complex waste or simple substrates is a very promising option for hydrogen production (Das & Vezirogul, 2001). The hydrogen is produced by anaerobic bacteria (e.g. *Enterobacter and Clostridium,*) on carbohydrate rich substrates (Manish & Banerjee, 2008; Ni *et al.,* 2006; Kotay & Das, 2008). Hydrogen can also be produced from proteins and lipid-rich substrates but carbohydrates are preferred because of higher yields (Levin *et al*., 2004; Claassen *et al.,* 1999).

The microorganisms degrade these organic compounds to gain both energy and carbon. Thus, glucose is degraded to two moles of pyruvate which is further converted to various end products, hydrogen being one of them. Strict anaerobes can produce H_2 from two major breakpoints during degradation of glucose, i.e. from NAD(P)H by

glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and from pyruvate ferredoxin oxidoreductase (PFOR) (Jones, 2008). The principal H_2 pathway is through PFOR because of thermodynamics hindrance of re-oxidizing NADH (Figure 13) (Jones, 2008).

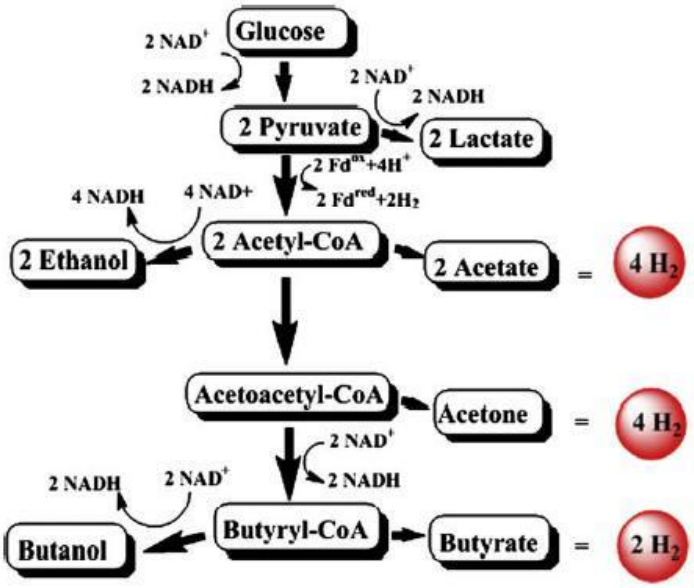

Figure 13 Anaerobic fermentation of clostridium. A simplified metabolic pathway (Chin 2003).

As previously mentioned, glucose can be degraded to various end products. If glucose is only degraded to acetate and CO_2, maximum yields of hydrogen (4 mol hydrogen per mole glucose) can be obtained (Argun *et al*., 2008):

$$\mathbf{C_6H_{12}O_6 + 2H_2O \rightarrow\ 2CH_3COOH + 4H_2 + 2CO_2\ (5)}$$

If butyrate is the only end product the theoretical maximum yield is 2 mols hydrogen per mol glucose, (Levin *et al*., 2004):

$$\mathbf{C_6H_{12}O_6 \rightarrow CH_3\ CH_2\ CH_2COOH + 2H_2 + 2CO_2\ (6)}$$

If other end products are produced, e.g. ethanol and lactate, it is unfavorable for hydrogen production and is not associated with the production presented in the following equations (Koskinen *et al*., 2008b):

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3CH_2OH + 2HCO_3 + 2H \quad (7)$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH + 2H \quad (8)$$

Many microorganisms produce a mixture of end products, both oxidized (acetate, and CO_2) and reduced (lactate, ethanol, butyrate). Thus, the yields of hydrogen production are depended on the microorganisms involved and also on the various environmental conditions they grow in. Known factors that influence H_2 production are hydrolytic retention time (HRT), pH, partial pressure of hydrogen and temperature. For optimum conditions, pH should be between 5 and 6, the HRT is 0.5 days from wastewater, but may vary with different substrates (Ni *et al.,* 2006; Kirtay, 2011). The partial pressure of hydrogen plays significant role in hydrogen production and is dependent on several factors, most importantly temperature. It is a well known phenomenon that the low H_2 yields observed by mesophilic and moderate thermophilic bacteria are because hydrogen production at low temperatures is thermodynamically unfavorable (Jones, 2008; Hallenbeck, 2009). At high temperatures, the influence of the partial pressure of H_2 is less on the key enzymes (hydrogenases) 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 that microorganisms growing at lower temperatures direct their end product formation to other reduced products. At low temperatures, elevated H_2 concentrations inhibit 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 favorable electron acceptors and consequently produce reduced products like EtOH, lactate, butyrate and alanine (Chou *et al*., 2008; Kengen *et al*., 2009)

2.5.2.5 Hydrogen production 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 *Thermoanaerobacter*, *Caldicellulosiruptor* and *Thermotoga*. Table 1 shows some of the most important data available from these genera in recent years. Clearly, highest yields are from the extremophiles *Caldicellulosiruptor* and *Thermotoga* although very good yields have been reported from *Thermoanaerobacter tengcongensis* (Soboh *et al*., 2004). *Clostridium* species has also been investigated for hydrogen production capacity (Levin *et al*., 2006; Geng *et al*., 2010; Magnusson *et al*., 2008; Almarsdottir *et al*., 2010).

Table 1 Hydrogen production from sugars

Organisms	Substrate	Cultivation method	H2 yield (mol/mol hexose)	Temp (C°)	Reference
Thermotoga maritima	Glucose	Batch	4	80	Schroder *et al.*, 1994
Thermotoga neapolitana	Glucose	Batch	3,9	77	Munro *et al*., 2009
Thermoanaerobacter tengcongensis	Glucose	Continuous	4,0	70	Soboh *et al*., 2004
Caldicelluloiruptor ownsenis (OL)	Glucose	Continuous	3,8	70	Zeidan & van Niel, 2010
Caldicelluloiruptor saccharolyticus	Glucose	Batch	2,7	70	Kadar *et al*., 2004
Pyrococcus furiosus	Maltose	Continuous	2,9	98	Schicho *et al.*, 1993
Closridium AK_{14}	Xylose	Batch	2,5	50	Almarsdottir *et al.*, 2010

2.5.2.6 Hydrogen production from complex biomass

Available data on hydrogen production from complex biomass has increased enormously in the last three years. Complex biomass, such as food waste and lignocellulosic agricultural residues (e.g. corn stover, wheat straw, cellulose waste water) have been used for thermophilic biohydrogen production in both laboratory and pilot scale. Selected data from recent literature is from pure cultures from thermophilic bacteria shown in Table 2. Highest yields and rates are from the study of Kádár and co-workers 2004 where H_2 yield is 3.7 mol/mol hexose (Akhtar & Amin, 2010; Kirtay, 2006; Kádár *et al*., 2004; Ni *et al*., 2006:).

Table 2 Hydrogen production from complex biomass.

Feedstock	Culture	H_2 yield mol/mol hexose	Temp. (C°)	References
Starch	*Thermococcus kodakaraensis*	3,3	85	Kanai *et al*., 2005
Sweet sorghum	*Caldicellulosiruptor saccharolyticus*	2,6	72	Ivanova *et al.*, 2009
Corn stover	*Thermoanaerobectrium thermo*	2,2	60	Cao *et al*., 2009
Miscanthus Hydrolysate	*Caldicellulosiruptor saccharolyticum*	3,4	70	de Vrije *et al*., 2009
Paper sludge	*Caldicellulosiruptor saccharolyticum*	3,7	70	Kádár *et al.*, 2004
Miscanthus hydrolysate	*Thermotoga neapolitana*	3,2	80	de Vrije *et al*., 2009

2.6 Thermophilic bacteria

In recent years, thermophilic anaerobic bacteria have gained increased attention as potential ethanol and hydrogen producing microorganisms. Thermophilic bacteria can be divided into several categories based on optimal growth temperatures, e.g. moderate thermophiles (T_{opt} from 45 to 55°C), true thermophiles (T_{opt} from 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 compared to mesophiles (Brock, 1986).

Studies of heat-resistant enzymes have shown that their amino acid sequences are virtually identical to enzymes that have mesophilic origin except for a few amino acids. By replacing one or several amino acids at key points in the enzyme can change the structure of the enzyme so that it can tolerate high temperatures. Many extreme thermophilic bacteria produce various substances which protect their proteins from the heat (Madigan *et al*., 2003). Many enzymes from thermophilic and extremethermophilic bacteria have been used in biotechnology. An example of this is Taq polymerase which was isolated from the bacterium *Thermus aquaticus*. Taq polymerase is very important in DNA duplication and is important tool in molecular biology (Madigan *et al*., 2003).

The habitats of thermophilic bacteria can vary widely, e.g. from hot springs, soils exposed to direct sunlight, heated industrial water, water-cooling towers and other manmade structures as well as in mines and deep surfaces (Koskinen *et al*, 2008b; Drent *et al*., 1991; Kanso *et al*., 2010; Perry, Staley, & Lory, 2002).

2.6.1. Thermophilic bacteria producing ethanol and hydrogen

There are relatively few genera of thermophiles that include bacteria with good hydrogen and ethanol producing capacities. Among good hydrogen producing bacteria are *Caldicellulosiruptor* and *Thermotoga* and the archaeons *Thermococcus* and *Pyrococcus* (Balk *et al*., 2002; Fardeau *et al*., 1997; Bredholt *et al*., 1999; Hamilton-Brehm *et al*., 2010; Kanai, *et al*., 2005; Verhaart, *et al*., 2010) but good ethanol producers are bacteria that belong to the genera *Clostridium, Thermoanaerobacter* and *Thermoanaerobacterium* (Collins, *et al*., 1994; Demain, *et al*., 2005; Sveinsdottir *et al*., 2009; Koskinen *et al*., 2008a, b). The amount of data concerning ethanol and hydrogen producing capacity of

these bacteria varies to a great extent. Much data is not on the efficiency of these bacteria to produce biofuels but merely on phylogenetic status and basic physiological properties. Most data is on rates and yields from monosugars but lately, also from hydrolysates from lignocellulosic biomass. Below, the most efficient ethanol and hydrogen producing thermophilic known today are described.

2.6.1.1 Clostridium

The genus *Clostridium* belongs to the family Clostridiaceae, order Clostridiales, class Clostridia and phylum Firmicutes. The genus contains a very diverse group of bacteria as shown by a phylogenetic analysis of Collins and co-workers (1994) where *Clostridium* species were compared to species belonging to the genus and to related taxa (Collins *et al*., 1994). It appears 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. 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. This is one of the main reasons for extensive research on biofuel production from complex biomass by *Clostridium* bacteria (Canganella & Wiegel, 1993; Carreira & Ljungdahl, 1993). Two of thermophilic Clostridia, *C. thermocellum* and *C. thermohydrosulfuricum* (now *Thermoanaerobacter thermohydrosulfuricum*) have attracted the most attention for biofuel production (Demain *et al*., 2005). Several cellulose-degrading enzymes form a structure called cellulosome, located and embedded on the external surface of the cell membrane (Demain *et al*., 2005).

2.6.1.2 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. The genus consists today of nine validly described species; *T.*

aciditolerans, *T. aotearoense*, *T. saccharolyticum*, *T. thermosaccharolyticum*, *T. thermosulfurigenes*, *T. xylanolyticum*, *T. fijiensis*, *T. polysaccharolyticum and T. zeae* (Cann *et al*., 2001; Collins *et al*., 1994; Kublanov *et al*., 2007; Lee *et al*., 1993; Liu *et al*., 1996).

Thermoanaerobacterium species are known for their abilities to convert carbohydrates to various end products like acetate, ethanol, lactate, hydrogen and CO_2. Some species have shown promising biofuel production capacities (Ren *et al*., 2008; 2009; 2010; Romano *et al*., 2010; Sveinsdottir *et al*., 2009) and *T. saccharolyticum* has been genetically engineered and then genes for both acetate (acetate kinase) and lactate (lactate dehydrogenase) formation has been knocked out (Shaw *et al*., 2008).

2.6.1.3 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 ethanol, acetate and lactate as major volatile end products (Lee *et al*., 1993).

Most species have broad substrate range and can degrade both pentoses and hexoses but not cellulose. 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; see also DSMZ and references there in). Most species produce ethanol and hydrogen as well as lactate, and in some cases alanine as end products (Fardeau *et al*. 1996). The type species, *Thermoanerobacter ethanolicus* and several other species within the genus have been extensively studied for ethanol production (Fardeau *et al*., 1996; Georgieva & Ahring, 2007; Georgieva *et al*., 2008; Lacis & Laword 1988a,b; Lamed & Zeikus, 1980a, b). Hydrogen production is usually low compared to ethanol by *Thermoanerobacter* although *T. 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).

2.6.1.4 Caldicellulosiruptor

The genus *Caldicellulosiruptor* was first proposed in 1994 by Rainey and coworkers on the basis of characteristics of a strain they isolated, *Caldicellulosiruptor saccharolyticus*

(Rainey *et al*., 1994). 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* **(**Bredholt *et al*., 1999; Hamilton-Brehm *et al*., 2010; Huang *et al*., 1998; Miroshnichenko *et al*., 2008; Mladenovska *et al*., 1995; Onyenwoke *et al*., 2006; Rainey *et al*., 1994; Yang *et al*., 2010**)**. All species are extremely thermophilic, cellulolytic, non-spore-forming anaerobes isolated from geothermal environments such as hot springs and lake sediments (Rainey *et al*., 1994; Yang *et al.*, 2010).

Caldicellulosiruptor species have relatively broad substrate spectrum capable to utilize e.g. cellulose, cellobiose, xylan and various pentoses and hexoses. Extreme thermophiles, have been shown to have superior hydrogen production yields and rates compared to mesophiles and produce few other byproduct besides acetate which makes *Caldicellulosiruptor* species excellent candidates for hydrogen production. *C. saccharolyticus* and *C. owensis* have been extensively studied for hydrogen production using sugar and hydrolysates from lignocellulosic biomass (Kadar *et al*., 2004; de Vrije *et al*., 2007; Zeidan & van Niel, 2010).

2.6.1.5 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 T. naphthophila*, *T. neapolitana*, *T. petrophila*, *T. subterranean* and *T. thermarum* **(**Balk *et al*., 2002; Fardeau *et al*., 1997; Huber *et al*., 1986; Jannasch *et al*., 1988; Jeanthon *et al*., 1995; Ravot *et al*., 1995; Takahata *et al*., 2001; Wiendberger *et al*., 1989**)**. These rod shaped bacteria are anaerobic, extremophilic that are characterized by an outer sheetlike structure called toga. (Huber *et al*., 1986; Jannasch *et al*., 1988). Most species have been isolated from deep environments, with high temperatures and pressure like from 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 various sugars, mainly to acetate, CO_2 and hydrogenbut do not have cellulolytic activity. Most strains have shown the property of reducing pyruvate to alanine from sugar fermentation (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).

2.6.1.6 Other thermophilic bacteria producing H_2 and EtOH

Apart from the above mentioned genera the capacity to produce ethanol and hydrogen has been reported for many other genera. Examples are species within *Caloramator, Caldanaerobacter, Caldanerobius* and the archaeons *Thermococcus* and *Pyrococcus* (Chrisostomos *et al*., 1996; Kozina *et al*., 2010; see also DSMZ and references there in).

3. The purpose of this study

This study is based on earlier projects at the University of Akureyri, supervised by Dr. Jóhann Örlygsson. The two strains investigated originate from the Krafla area (Víti) in North East Iceland and from Grensdalur in SW-Iceland.

The main objective of the first project was to investigate the thermophilic bacterium *Clostridium* AK_{14}, a powerful hydrogen producer. Optimal growth conditions were observed, fermentation of glucose to various end product, effect on increased substrate (glucose) concentration on end product formation, its substrate spectrum and its ability to produce hydrogen from hydrolysates made of lignocellulosic biomass (see Chapter 5; manuscript I).

The second project, focused on ethanol production form sugars and lignocellulosic biomass by *Thermoanaerobacterium* strain AK_{17}. Various environmental factors (hydolysate concentrations, chemical pretreatment, and amount of enzymes) were investigated in detail to maximize ethanol production from lignocellulosic biomass. Also, the effect of known inhibitory compounds (furfural and hydroxymethylfurfural) on ethanol production were investigated (see chapter 6; .manuscript II).

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5 Manuscript I

Hydrogen production from sugars and complex biomass by *Clostridium* species AK_{14}, isolated from Icelandic hot spring

Hydrogen production from sugars and complex biomass by *Clostridium* species, AK_{14}, isolated from Icelandic hot spring

ARNHEIDUR RAN ALMARSDOTTIR,
ALICJA TARACEVIZ
INGOLFUR GUNNARSSON
AND
JOHANN ORLYGSSON

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Abstract

The hydrogen production ability of strain AK_{14} , a moderate thermophilic bacterium was studied. According to 16S rRNA gene sequence analysis the strain belongs to genus *Clostridium* but is most closely related to *Anaerobacter polyendosporus* (95.1% similarity). Growth of strain AK_{14} was observed at temperatures between 42 and 52°C with optimal growth at 50°C. Optimum pH for growth was at pH 6.5 but growth was observed from pH 4.5 to 7.5. Fermentation of glucose resulted in the production of acetate and butyrate (major) and ethanol (minor) as well as hydrogen and carbon dioxide. Effect of increased substrate (glucose) concentration was investigated and good correlation was observed between increased substrate loadings and end product formation up to 50 mM. At ≥ 50 mM glucose concentrations, end product formation levelled off and the substrate was not completely degraded. Also the pH at the end of fermentation dropped from 7.0 (control without glucose) to 5.3 (at ≥ 50 mM glucose). The ability to utilize various carbon substrates was tested, with positive growth observed on xylose, glucose, fructose, mannose, galactose, starch and xylan. The end products in all cases were the same as for glucose. By varying the liquid to gas phase during glucose fermentation in a batch a clear correlation was found between increased acetate, butyrate and hydrogen production at low gas-to-liquid conditions and less ethanol. Amount and identification of the end products from hydrolysates made of lignocellulosic (5.0 g L^{-1} ([dw])) biomass (cellulose from Whatman paper), newspaper, grass (*Phleum pratense*), barley straw (*Hordeum vulgare*), and hemp (stem and leaves of*Cannabis sativa*), was investigated. The biomass was pretreated with either a weak acid (HCl) or a weak base (NaOH) as well as enzymes (Celluclast® and Novozyme 188). The strain produced the most hydrogen (8.5 mol H_2 g^{-1} VS) from cellulose hydrolysate but less from paper and lignocellulosic biomasses (between 0.26 to 3.60 mol H_2 g^{-1}). The hydrogen production from lignocellulosic biomass was however enhanced significantly by acid and base pretreatment with the highest production from grass (6.23 mol H_2 g^{-1} VS). Other end products were acetate, butyrate and ethanol.

Keywords: hydrogen, hot spring, *Clostridium*, carbohydrates, lignocellulose

Yfirlit

Framleiðsla á vetni úr sykrum og flóknum lífmassa með Clostridium tegund, AK_{14}, einangraðri úr íslenskum hver

Vetnisframleiðsluhæfileiki bakteríustofnsins AK_{14} sem er hitakær baktería einangruð úr íslenskum hver var rannsökuð. Skyldleikarannsókn með 16S rRNA greiningu leiddi í ljós að stofninn tilheyrir ættkvísl *Clostridium* en var skyldust *Anaerobacter polyendosporus* (95.1% skyldleiki). Stofninn vex við hitastig á milli 42 til 52°C en hámarksvöxtur var við 50°C. Kjörsýrustig var við pH 6.5 en mælanlegur vöxtur var við sýrustig á milli pH 4.5 og 7.5. Niðurbrot á glúkósa leiddi aðallega til framleiðslu á ediksýru og smjörsýru auk vetnis en einnig var smávægileg framleiðsla af etanóli. Áhrif mismunandi upphafsstyrks af glúkósa á móti framleiðslu lokaafurða var rannsakaður hjá bakteríunni. Við lágan upphafsstyrk glúkósa voru bein tengsl á milli aukins styrks hvarfefnis og myndefna, þ.e. aukning á glúkósa leiddi til línulegrar aukningar á lokaafurðum. Hins vegar þegar styrkur glúkósa náði 50 mM kom í ljós greinleg hindrun og glúkósinn var ekki brotinn fullkomlega niður. Sýrustigið í lok gerjunar lækkaði einnig með auknum styrk glúkósa en það var 7.0 án glúkósa og fór niður í 5.3 við 50 mM upphafsstyrk glúkósa en lækkaði ekki frekar við hærri glúkósastyrk. Hæfileiki stofnsins til að brjóta niður mismunandi kolefnisgjafa leiddi í ljós að hann brýtur niður xýlósa, glúkósa, frúktósa, mannósa, galaktósa, sterkju og xýlan. Lokaafurðir við niðurbrot þessara hvarfefna var í öllum tilfellum ediksýra, smjörsýra, etanól, vetni og koltvísýringur. Með því að nota mismunandi hlutfall á milli gas og vökvafasa við niðurbrot á glúkósa kom í ljós að hlutfall ediksýru, smjörsýru og vetnis var mest þegar gasfasinn var hlutfallslega stór miðað við vökvaræktina en minna var framleitt af etanóli. Vöxtur var einnig athugaður í 0.5% (wv^{-1}) "hýdrólýsötum" sem gerð voru úr sellulósa (Whatman pappír), dagblaðapappír, vallarfoxgrasi (*Phleum pratense*), hálmi úr byggi (*Hordeum vulgare*), og hampi (stilkar og laufblöð (*Cannabis sativa*)). Lífmassinn var formeðhöndlaður með veikri sýru (HCl) eða veikum basa (NaOH) sem og ensímum. Mest vetni framleiddi stofninn úr sellulósa (8.5 mól $H_2g^{-1}VS$) en mun minna úr öðrum lífmassa (0.26 to 3.60 mol H_2g^{-1} VS). Hins vegar var hægt að auka þessa framleiðslu til muna með því að formeðhöndla lífmassann og hæsta gildið fékkst úr vallarfoxgrasi eða 6.23 mol H_2g^{-1} VS. Aðrar lokaafurðir voru þær sömu og áður, þ.e. ediksýra, smjörsýra og etanól.

Introduction

Depletion of fossil fuel reserves together with global climate changes caused by increase of CO_2 in the atmosphere are leading to the search for alternative, renewable, carbon-free energy sources (Rittmann, 2008). Biohydrogen has a great potential as a clean renewable energy carrier since it has a high energy content, producing water as the sole end product after combustion (Das & Veziroglu, 2001). Additionally molecular hydrogen has a high energy yield (120 MJ/kg) which is almost three times higher than most hydrocarbon fuels (Kapdan & Kargi, 2006). Lignocellulose is the most abundant biopolymer on earth and the main component of plant biomass. Fermentation of lignocellulosic biomass (e.g. wood, straw, grass and waste material from for example agricultural and municipal activities and the pulp and paper industry) therefore constitutes an interesting alternative for the production of biohydrogen (Balat *et al*., 2008). Hydrogen can be produced microbiologically through fermentation from various starch- and sugar-based materials (Hawkes *et al*., 2002), including lignocellulosic biomass. The thermal pretreatment of lignocellulosic biomass most often results in two main streams of substrates, i.e. a solid fraction consisting of cellulose (hexose; glucose) (Klinke *et al*., 2002) and a liquid phase (hydrolysate) mainly consisting of hemicellulose (pentose; xylose and arabinose) (Bercier *et al*., 2007). Producing biofuels from ligncellulosic biomass economically requires that all the main carbohydrates are converted to high hydrogen production through fermentation routes (Olsson & Hahn-Hagerdahl, 1996). Hydrogen production by fermentation has been receiving increased attention and high production rates have been achieved in bacterial fermentations (Wu *et al*., 2006; Koskinen *et al*., 2008a). Thermophiles have many advantages compared to mesophilic microorganisms in hydrogen production because of their high growth rates and their ability to degrade a broad range of substrates and their high hydrogen yields (Sommer *et al*., 2004, van Groenestijn *et al*., 2002; Zeidan & van Niel, 2010). Additionally, high temperature favours the kinetics and stoichiometry of hydrogen-producing reactions (van Groenestijn *et al*., 2002; Wang & Wan, 2009). With increasing temperatures, hydrogen-producing metabolism becomes thermodynamically more favourable (Stams, 1994) and less affected by the partial pressure of hydrogen in the liquid phase (van Gooenestijn *et al*., 2002). Additionally, many thermophiles have a narrower spectrum of fermentation end products compared to mesophiles (van Niel *et al*., 2003).

Hydrogen production from carbohydrates is considered to be produced mainly through acetate and butyrate production:

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

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

Other end products common in fermentation are ethanol and lactate, which both result in no hydrogen production (Thauer *et al*., 1977; Wiegel, 1980). Thus, the most feasable fermentation of carbohydrates is towards acetate and butyrate and not to reduced electron scavenging products like ethanol and lactate. The highest yields observed for hydrogen production are from the extreme thermophile *Caldicellulosiruptor saccharolyticus* under gas sparging (de Vrije *et al*., 2007). The strain produced only acetate and H_2 + CO_2 with hydrogen yields of 3.6 mol-H_2 mol-glucose^{-1}. *Thermotoga elfii* has been reported to produce 3.3 mol H_2 mol-glucose^{-1} (van Niel *et al*., 2002) and an enrichment culture from Icelandic hot spring, 3.2 mol-H_2 mol-glucose^{-1} (Koskinen *et al*., 2008a).

Hot springs are a potential source for hydrogen producing microorganisms (Wiegel & Ljungdahl, 1981; Sommer *et al*., 2004; Orlygsson & Baldursson; 2007, Koskinen *et al*., 2008b). In this study a moderate thermophilic fermentative bacterium efficient in hydrogen and acetate/butyrate production was studied. Hydrolysates from various types of complex lignocellulosic biomass were used to test the hydrogen production performance of the bacterium. Optimal conditions for hydrogen production in terms of initial substrate concentration and the partial pressure of hydrogen (pH_2) were investigated as well as the main substrate spectrum capacity of the strain.

Materials and methods

Media, isolation and strain identification

The medium (BM medium) composition and preparation was done according to Orlygsson & Baldursson (2007) and Sveinsdóttir *et al*., (2009). Isolation and full 16S rRNA gene sequence analysis for the strain were described earlier (Orlygsson & Baldursson, 2007).

Determination of growth

Cell concentration was determined by measuring absorbance at 600 nm by a Perkin Elmer spectrophotometer. Maximum (specific) growth rate (μ_{max}) for each growth experiment was derived from the absorbance data (OD_{600}) using the equation: $\ln(x/x_0) = (\mu)(t)$, where x is the measurement of optical density of the culture, x_0 is the initial optical density of the culture, t is the elapsed time and μ denotes the maximum growth rate. All experiments were done in duplicates and average values are reported.

Determination of pH_{opt} and T_{opt}

To determine the strain's optimum pH for growth the pH was set to various levels in the range of 3.0 to 9.0 with increments of 0.5 pH units. The experimental bottles were supplemented with acid (HCl) or base (NaOH) to adjust the pH accordingly. To determine the optimum temperature for growth the incubation temperature varied from 30°C to 60°C. For the pH optimum determination the strain was cultivated at 50°C and for the temperature optimum determination the pH was 6.5. Control samples did not contain glucose. Optimal pH and temperature were thereafter used in all experiments performed.

Effect of substrate concentration

The effect of increased glucose concentration was tested on strain AK_{14}. Initial glucose concentration varied between 5 to 400 mM. Control samples did not contain glucose. Optical density was measured at the beginning and at the end of the incubation period (5 days) to determine the growth. Glucose, hydrogen, volatile fatty acids (VFA) and ethanol were measured as well as the final pH in the experimental bottles. Experiments were done in 120 mL serum bottles with 50 mL of liquid medium.

Substrate utilization

The ability of strain AK_{14} to utilize different substrates was tested using the BM medium (Orlygsson & Baldursson 2007) supplemented with various filter sterilized substrates (20 mM or 2 g L^{-1}). The substrates tested were: xylose, ribose, arabinose, glucose, fructose, galactose, mannose, sucrose, lactose, lactate, formate, succinate, malate, pyruvate, oxalate, crotonate, glycerol, inositol, starch, cellulose, xylan, sorbitol, pectin, casamino acids, peptone, beef extract, tryptone, alanine, aspartate, glycine, glutamate, serine, theronine, histidine and cysteine. Growth was observed by increase in optical density which was measured at the beginning and at the end of the incubation period (5 days). Where growth was detected, hydrogen, volatile fatty acids and ethanol were analysed. Experiments were done in 23 mL serum bottles with 10 mL of liquid medium.

Effect of gas-liquid volume ratio on hydrogen production

The influence of partial hydrogen pressure (ρH_2) on hydrogen production was investigated with different ratios of liquid and gas phases on glucose (20 mM). The liquid phase varied from 2 to 90 mL in serum bottles with a total volume of 120 mL; thus, the liquid/gas volume ratio varied from 0.016 to 3.00. After 5 days of incubation, glucose content and the end product formation (hydrogen, acetate, butyrate and ethanol) were measured.

Pretreatment of biomass and hydrolysate preparation

Hydrolysates (HL) were made from different biomasses: Whatman filter paper (cellulose), hemp (*Cannabis Sativa*) – leaves and stem fibres (Fedora cultivar), newspaper with ink (NPi), barley straw (BS) (*Hordeum vulgare L.*) and grass (*Phleum pratense L.*). Whatman paper consists of 99% cellulose and was therefore used as a control sample. The preparation of the hydrolysates was according to Sveinsdottir *et al*. (2009) yielding a final dry weight of 25 g L^{-1}. Chemical pretreatment in the form of acid (0.75% H_2SO_4) or base (0.75% NaOH) (control was without chemical pretreatment) were done before autoclaving for 30 minutes (121°C). After heating, the bottles were cooled down to room temperature and the pH adjusted to 5.0 by adding either HCl or NaOH. Two enzymes were added to each bottle, Celluclast® and Novozyme 188 (1 mL of each; 0.25% vol/vol), and incubated in a water bath at 45°C for 68h. After the enzyme treatment the pH was measured again and adjusted to the pH optimum of the strain. The solutions were then filtered into sterile bottles to collect the hydrolysates.

Fermentation of hydrolysates

Fermentation of carbohydrates present in hydrolysates by strain AK_{14} was done in 23 mL serum bottles. The medium (8 mL) was supplemented with 2 mL of hydrolysate in each sample (total liquid volume 10 mL), giving a final concentration of 5.0 g L^{-1}. The control sample contained no hydrolysate. The concentration of salts, vitamins and trace elements was kept the same as in the medium without any addition of hydrolysate.

Analytical methods

Hydrogen, ethanol and volatile fatty acids were measured by gas chromatograph as previously described (Orlygsson & Baldursson, 2007). Glucose was determined by injecting 20 µL samples filtered through a 0.2 µm PTFE filter onto a Shimidzu HPLC equipped with a Thermo Hypersil Gold aQ reverse phase column (4.6 x 250 mm), RID-10A Refractive Index Detector, a SPD-20A UV-Vis detector at 190 nm and a LC-20AD solvent pump delivering 50 mM NaH_2PO_4 at a flow rate of 1 mL min^{-1}. Glucose was also analysed by slight modification of the method from Laurentin & Edwards (2003). A liquid sample (400 µL) was mixed with 2 mL of anthrone solution (0.2% anthrone in 72% sulphuric acid). The sample was boiled for 11 minutes and then cooled down on ice. Optical density was then measured at 640 nm.

Results and discussion

Hydrogen- and ethanol-producing bacteria have gained much attention in recent years because of increased interest in renewable energy sources. High yields of hydrogen from sugar fermentation have been reported using the extremophiles *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii*, e.g.3.3 to 3.6 mol-H_2 mol-carbohydrate^{-1} (van Niel *et al*., 2002; de Vrije *et al*., 2007) and an enrichment culture from Icelandic hot springs, 3.2 mol-H_2 mol-glucose^{-1} in a semi-continuous batch reactor (Koskinen *et al*., 2008a).

Identification and phenotypic characteristics

The isolates had a rod shape, with a length from 0.5 to 2.5 μm. The cells had a rounded end and did not produce spores. They occurred singly or in pairs, though rarely in chains. The cells stained gram-positive. 16S rRNA gene sequence analysis revealed that strain AK_{14} is a member of the genus *Clostridium* Cluster I (Orlygsson & Baldursson, 2007). The closest phylogenetic relative was *Anaerobacter polyendosporus* with 95.1% similarity of the 1425 bp analysed. Other species that were most closely related were *Clostridium bovipelis* (93.7%), *Clostridium tertium* (93.0%), *Clostridium sartagoforme* (92.9%), *Clostridium putrefaciens* (92.2%) and *Clostridium disporicum* (92.0%). All these species are anaerobic mesophiles fermenting sugars and/or amino acids. *Anaerobacter polyendosporus* and *Clostridium disporicum* produce five and two spores, respectively (Duda *et al*., 1987, Horn, 1987), but no spores have been detected from strain AK_{14} under any of the growth conditions tested. The most closely thermophilic bacterium related to AK_{14} is *Clostridium thermobutyricum* (89.9%) isolated from horse manure and which produces butyrate from sugar fermentation as the main end product (Wiegel *et al*., 1989).

The strain was isolated from Grensdalur in SW-Iceland (Orlygsson & Baldursson, 2007). The temperature of the hot spring it originated from was 51.0°C and the pH 7.8. The strain grows in a very narrow temperature range (42 to 52°C), with optimal growth at 50°C (generation time 0.54h). Optimum pH for growth was pH 6.5 (generation time; 0.56h), though growth was observed from pH 4.5 to 7.5.

End product formation from glucose and xylose

The phenotypic properties of AK_{14} were consistent with those of many saccharolytic *Clostridium* species capable of degrading various carbohydrates to volatile fatty acids,

ethanol and H_2 plus CO_2. End product formation from glucose and xylose (20 mM each) resulted in the production of acetate and butyrate (as main products) and ethanol (minor product) together with H_2 and CO_2:

20 mM Glucose → 2.8 mM EtOH + 7.5 mM Acetate + 10.7 mM Butyrate + 30.5 mM H_2 + 31.7 mM CO_2

20 mM Xylose → 0.0 mM EtOH + 5.9 mM Acetate + 10.9 mM Butyratre + 35.3 mM H_2 + 27.7 mM CO_2

The carbon recovery on glucose and xylose were 79.3 and 83.1%, respectively. Lactate was not analysed in this study but has been shown to be a minor product from glucose fermentation by AK_{14} (Orlygsson & Baldursson, 2007). Additionally, some of the substrate is converted into biomass but Kim *et al*., (2006) have reported that this value is approximately 11%.

All species in the *Clostridium* cluster I (*Clostridium* sensu stricto) produce butyrate (Collins *et al*., 1994; Andreesen *et al*., 1989). Butyrate production is however relatively uncommon among thermophilic bacteria and has only been reported as the main product for *Clostridium thermobutyricum* (Wiegel *et al*., 1989; Canganella *et al*., 2002). The fact that AK_{14} grows at temperatures that are on the boundary between mesophilic and thermophilic perhaps explains this property of the strain. Indeed, enrichment studies from various geothermal areas in Iceland, ranging in tempearatures from 50 to 75°C, revealed that butyrate production was common only from the lower (50°C) temperatures enrichments (Orlygsson *et al*., 2010).

Carbon source utilization pattern

One of the major reasons for using thermophilic bacteria for hydrogen production from lignocellulosic material is their ability to degrade a broad range of carbohydrates present in the biomass. AK_{14} grew on various types of carbohydrates as the sole carbon and energy source (Table 1). Of the three tested pentoses this strain only degraded xylose but all four hexoses tested (glucose, frucose, galactose, mannose) were degraded as well as the disaccharide sucrose thought not lactose. Additionally, the strain degraded starch and xylan. All of the other substrates tested were not degraded. The end products formed on all substrates were, as for glucose and xylose: acetate, butyrate, H_2 + CO_2 (major) and ethanol (minor).

Effect of environmental parameters on fermentation

Effect of partial pressure of hydrogen

The change in the partial hydrogen pressure (pH_2) is known to affect the end product formation by anaerobic bacteria such that at a high pH_2 more reduced products like ethanol and lactate are formed rather than acetate, butyrate and H_2 (Nath & Das, 2004, Hawkes *et al.*, 2002). This was indeed observed for strain AK_{14} in a previous study (Orlygsson & Baldursson, 2007) where the theoretical yield of hydrogen increased from 38 to 55% when the liquid/gas volume ratio was changed from 3 to 0.02 (Figure 1). In this case it is assumed that the theoretical yield is 4 moles of hydrogen per mole degraded hexose and acetate is the only volatile end product (Nandi & Sengupta, 1998; Hawkes *et al*., 2002). This experiment was repeated in the present investigation but now volatile end products and ethanol formation were also analysed to gain further insight into the metabolism of the bacterium. Again, similar yields of hydrogen were observed, varying from 1.6 to 2.6 mol-H_2 mol glucose^{-1} (40 to 65% of theoretical yield). As expected, lower H_2 yields followed the decrease in acetate and butyrate formation as against an increase in ethanol production (Figure 1).

Using the fermentation data from the lowest and highest L/G ratios the following equations are observed:

1.0 Glucose → 0.06 EtOH + 0.59 Acetate + 0.75 Butyrate + 2.60 H_2 + 2.15 CO_2 (low L/G (0.05) ; Eq.1)
1.0 Glucose → 0.28 EtOH + 0.39 Acetate + 0.58 Butyrate + 1.60 H_2 + 1.63 CO_2 (high L/G (3.0) ; Eq.2)

It has been reported that butyrate to acetate (B/A) ratios are directly proportional to H_2 yields (Kim *et al*., 2006). The B/A ratios observed in batch cultures with different L/G ratios ranged from 1.3 to 1.5 on a molar basis. Therefore, in this study, 1.4 was used to characterize the stoichiometric reaction for the production of H_2 from glucose. This would result in a theoretical hydrogen yield of 2.36 mol-H_2/mol-glucose. This is in good agreement with the obtained result and fits between the results obtained in eq. 1 and eq. 2.

Effect of initial substrate concentrations

High initial substrate concentration may play an important role in hydrogen production rates and yields (Kumar & Das, 2001; Lacis & Lawford, 1988; van Ginkel & Sung, 2001, Sommer *et al*., 2004). This has been investigated for many bacteria, e.g. *Citrobacter* species where glucose loadings of 1, 5 and 25 g L^{-1} resulted in 2.5, 1.2 and 0.8 mol-H_2 mol-glucose^{-1} degraded (Oh *et al*., 2003). However, fermenting microorganisms can also have limited tolerance to increased substrate loadings (van Ginkel & Logan, 2005; Olsson & Hahn-Hägerdal, 1996). This was clearly observed in the present study when strain AK_{14} was cultivated at different initial concentrations of glucose varying from 5 to 400 mM (Table 2). More than 94% of the glucose was degraded at low (5, 10 and 30 mM) concentrations but much less at higher (≥ 50 mM) concentrations. This is also reflected in similar amounts of end products at high initial glucose concentrations compared to the lower substrate concentrations. The pH was measured after fermentation and decreased from low glucose loadings (pH 7.0) to high (≥ 50mM) loadings (pH 5.2 to 5.3). This, together with the fact that end product formation levels off at high substrate loadings, indicates that this inhibition is more likely to be caused by the low pH rather than the high substrate loadings.

End product formation from hydrolysates

Hydrogen production from lignocellulosic biomass has recently received increased attention. Several studies on thermophilic bacteria growing on untreated wastewater cellulose have shown yields between 0.82 and 1.24 mols-H_2 mol-glucose^{-1} equivalents (Magnusson *et al*., 2008; Liu *et al*., 2003). Co-culture studies of *Clostridium thermocellum* and *Thermoanaerobacterium saccharolyticum* on hydrogen production from microcrystalline cellulose resulted in 1.8 mol-H_2 mol-glucose^{-1} equivalents (Liu *et al*., 2008). Other studies on pretreated hydrolysates from lignocellulosic biomass have shown higher yields. Lalaurette *et al*., (2009) showed hydrogen yields of 1.64 mol-H_2 mol-glucose^{-1} equivalent from corn stover hydrolysates (pretreated with dilute sulphuric acid) by *Clostridium thermocellum.* Mixed culture studies (35 and 50°C) on similar biomass pretreated with steam explosion and dilute sulphuric acid resulted in 2.84 mol-H_2/mol-glucose equivalents (Datar *et al*., 2007).

When strain AK_{14} was grown on different hydrolysates of biomass (cellulose, hemp leaves, hemp stem fibres, newspaper, barley straw and grass) the highest hydrogen production resulted on cellulose (33.0 mmol L^{-1}), hemp stem (13.5 mmol L^{-1}) and grass (10.9 mmol L^{-1}) when no chemical pretreatment was used (Table 3). This represents 8.5, 3.5 and 2.9 mol H_2 g VS^{-1} of the corresponding biomass. Fermentation of hydrolysates from other biomass types resulted in lower hydrogen production. The volatile end products were, as observed on monosugars, acetate and butyrate (major end products) and ethanol (minor). Acid and base pretreatment did not enhance end product formation for cellulose hydrolysates, as was observed on other hydrolysate biomass types. This was mostly profound for the lignocellulosic biomass but less for paper. The hydrogen production from grass and straw was two and three times higher, respectively, with chemical pretreatment as compared with the untreated sample. A similar increase was observed on hemp leaves but less on hemp stems. In general there was little difference between using a base or an acid as pretreatment. Base pretreatment of grass and straw gave a slightly higher production but less for hemp and paper.

Glucose was analysed in the cellulose hydrolysate (without chemical treatment) after fermentation and measured 3.5 mM. Assuming that all glucose is released from pure cellulose during hydrolysis, an initial glucose concentration of 30.9 mM would be available for fermentation. Thus, assuming that 27.4 mM of glucose were degraded the end product formation can be compared to the pure glucose fermentation spectrum described earlier. The hydrolysate experiment was done with an L/G ratio of 0.76 compared to an L/G ratio of 1.00 (data from Figure 1). The stochiometry for pure glucose and the cellulose hydrolysate (HL) experiments are:

1.00 Glucose → 0.20 EtOH + 0.35 Acetate + 0.58 Butyrate + 1.60 H_2 + 1.84 CO_2 (glucose)
1.00 Glucose → 0.09 EtOH + 0.36 Acetate + 0.45 Butyrate + 1.78 H_2 + 1.35 CO_2 (HL)

The end product formation in the cellulose hydrolysate experiment was slightly higher except for ethanol and carbon recovery was 80%. The hydrogen yield on cellulose hydrolysate was 1.39 mol-H_2 mol-glucose^{-1} equivalent. This was higher than the results in many of the above mentioned studies (Liu *et al*., 2008; Lalaurette *et al*., 2009), which makes strain AK_{14} an interesting alternative as a hydrogen producer from lignocellulosic biomass. The lower hydrogen values obtained on other hydrolysates can be explained by

the lignin content (not measured) or by inefficient pentose fermentation that was released from the hydrolysis of the hemicellulose fraction of the lignocelluloses.

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FIGURES AND TABLES

Legends:

Figure 1. Effect of liquid/gas volume ratio on end product formation for strain AK_{14}. Ethanol (♦), Acetate (■), Butyrate (▲) and hydrogen (X).

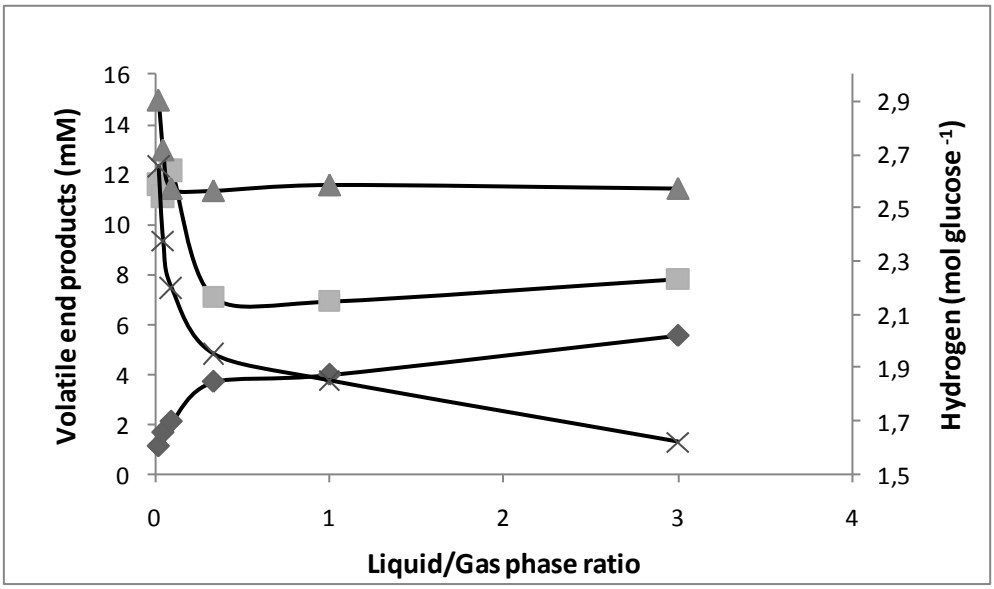

Table 1. End product formation from various substrates by strain AK_{14}. Values represent mean of two replicates.

Substrates	**End product formation (mmol/L)**			
	Ethanol	**Acetate**	**Butyrate**	**Hydrogen**
Yeast extract	1.6	1.8	1.2	1.2
Xylose	0.7	6.7	11.2	51.1
Glucose	3.2	9.2	10.8	44.8
Fructose	2.3	8.2	11.8	39.0
Galactose	1.0	9.5	13.1	31.2
Mannose	0.6	8.9	14.1	28.2
Sucrose	3.1	10.8	16.2	46.0
Starch	1.6	5.5	8.6	22.3
Xylan	1.0	2.8	2.3	3.9

Table 2. End product formation from different initial glucose concentrations. Also shown are glucose concentrations after fermentation and end pH values. Values represent mean of two replicates.

Initial glucose (mM)	**End product formation (mmol/L)**			**End glucose (mM)**	**End pH**
	Ethanol	**Acetate**	**Butyrate**		
0	1.6	2.4	1.0	0.0	7.0
5	3.1	3.8	1.0	0.3	6.6
10	5.0	9.8	3.1	0.3	6.4
30	6.6	12.9	5.5	0.4	5.9
50	6.7	11.5	15.8	10.3	5.2
100	5.9	10.6	20.0	55.9	5.3
200	6.3	11.3	17.2	159.5	5.3
400	7.0	14.1	16.6	357.5	5.3

Table 3. Production of end products from hydrolysates made from different biomass. Values represent mean of two replicates.

	End product formation (mmol/L)			
Biomass and pretreatement	**Ethanol**	**Acetate**	**Butyrate**	**Hydrogen**
Cellulose	0.6	8.6	11.9	33.0
Cellulose - acid	1.2	6.6	10.9	29.7
Cellulose- base	0.6	8.7	11.8	32.8
Hemp Stem	0.9	4.1	4.8	13.5
Hemp Stem - acid	0.8	4.5	7.1	17.8
Hemp Stem - base	0.3	5.0	6.2	16.7
Hemp Leaf	0.1	0.2	0.7	0.0
Hemp Leaf - acid	0.4	2.8	3.3	10.0
Hemp Leaf - base	0.2	1.8	2.3	6.4
Grass	1.0	2.9	4.4	10.9
Grass - acid	1.1	5.1	8.0	22.6
Grass - base	1.2	5.9	8.5	24.2
Paper	0.1	2.5	3.2	7.2
Paper- acid	0.4	3.0	4.0	10.7
Paper - base	0.1	0.5	1.0	2.6
Straw	1.0	2.0	3.0	6.5
Straw - acid	0.9	4.8	7.2	19.6
Straw - base	1.2	5.8	8.6	20.9

6 Manuscript II

Effect of environmental factors on the yields of ethanol production from lignocellulosic biomass by *Thermoanaerobacterium* AK_{17}

Submitted for publication

Effect of environmental factors on the yields of ethanol production from lignocellulosic biomass by *Thermoanaerobacterium* AK_{17}

ARNHEIDUR RAN ALMARSDOTTIR,
MARGRET AUDUR SIGURBJORNSDOTTIR,
JOHANN ORLYGSSON

Abstract

The ethanol production capacity from sugars and lignocellulosic biomass by *Thermoanarobacterium* strain AK_{17} was studied. The strain degrades various carbohydrates to, acetate, ethanol, hydrogen and carbon dioxide. Ethanol yields on glucose and xylose were 1.5 and 1.1 mol/mol sugars, respectively. Effect of increased initial glucose concentration on end-product formation and the substrate spectrum was investigated. The influence of various environmental factors (concentrations of hydrolysates, enzymes and acid/alkaline) on end-product formation from lignocellulosic hydrolysates as well as the effect of inhibitory compounds were investigated in detail. Optimization experiments for ethanol production at low hydrolysate concentrations (2.5 g L^{-1}); with 0.25% acid/alkali (v/v) and 0.1 mL g^{-1} enzyme concentrations resulted in 5.5 and 8.6 mM g^{-1} grass and cellulose, respectively. Inhibitory effects of furfural and hydroxymethylfurfural, revealed a total inhibition in end product formation from glucose at 4 and 6 g/L, respectively.

Keywords: Ethanol, hot spring, *Thermoanaerobacterium*, carbohydrates, lignocelluloses, hydrolysates

Introduction

Ethanol production from lignocellulosic biomass is an interesting alternative to first generation production of ethanol. The world's annual ethanol production from biomass has increased enormously in recent years. In the year 2000 the annual U.S. production was 1.63 billion gallons (U.S. International Trade Commission, 2008) but 10.6 billion gallons in 2009. Most of this increase is however from starch and sugar based biomass which has led to an intensive food versus fuel debate worldwide. Lignocellulosic biomass is the most abundant biomass available today and the estimated annual production in U.S. could exceed 90 billion gallons (Renewable Fuel Association, 2011). Lignocellulose is composed of cellulose, hemicellulose, and lignin and is more complex compared to starch and sugars. The hemicellulose and cellulose fraction of lignocellulosic material can be used as substrates for second generation ethanol production. However, expensive pretreatments are needed to segregate these fractions from lignin and hydrolyse them to monosugars for fermentation (Gírio *et al*., 2010). The main bottleneck for ethanol production from lignocelluloses is thus the pretreatment costs as well as lack of suitable microorganisms with broad substrate spectrum. Traditionally, the yeast *Saccharomyces cerevisae* has been used for first generation of ethanol production from starch and sugar based biomass. The wild type cannot degrade the pentoses present in the hemicellulosic fraction of the second generation ethanol production (lignocelluloses) and engineered strains seem to lack stability for large scale production (Lin & Tanaka, 2006).

Therefore, increased interest has been on the use of thermophilic bacteria with broad substrate range and high yields. Thermoanaerobes have gained attention after the oil crisis in the 1980's (Wiegel, 1980; Wiegel & Ljungdahl, 1981). Thermophilic bacteria within the genera of *Thermoanaerobacterium*, *Thermoanaerobacter* and *Clostridium* have been investigated and showed good ethanol yields and fast growth rates (Wiegel *et al.*, 1979; Lacis & Lawford, 1988; Sveinsdottir *et al.*, 2009; Orlygsson *et al.*, 2010;). There are several advantages of using these bacteria: the increased temperature deters contamination from mesophilic bacteria, possible self distillation of ethanol avoiding the generally low ethanol tolerance problem with those bacteria and broad substrate spectrum (Sommer *et al.*, 2004; van Groenestijn *et al.*, 2002). Most studies on thermophilic ethanol producing bacteria have been on simple sugar fermentation, both in batch and continuous cultures.

Several bacteria have shown good yields, e.g. between 1.5 to 1.9 mol-EtOH mol-hexose^{-1} degraded (Sveinsdottir *et al*., 2009; Lacis & Lawford, 1988).

Relatively few studies are on ethanol yields from hydrolysates made from lignocellulosic biomass. The present study focuses on a thermophilic bacterium, *Thermoanaerobacterium* AK_{17}, which has been shown to produce up to 1.5 mol-EtOH mol-glucose^{-1} and 1.1 mol-EtOH mol-xylose^{-1} as well as good yields from lignocellulosic biomass (Sveinsdottir *et al*., 2009). Additionally, continuous culture experiments on glucose have been done showing similar yields (Koskinen *et al*., 2008). Data on various environmental factors, e.g. concentrations of substrates (sugars and hydrolysates), enzymes, acids and alkalis for optimal yields of ethanol from cellulose and grass hydrolysates are presented. Finally, the inhibitory effects of furfural and hydroxymethylfurufural on the strain's end product formation are investigated.

Materials and methods

Medium

The medium (BM medium) was prepared according to Sveinsdottir *et al*. (2009). Isolation and full 16S rRNA gene sequences analysis for the strain was previously described (Orlygsson and Baldursson, 2007).

Effect of substrate concentration

The effect of glucose concentration on strain AK_{17}, by varying the concentration from 5 to 200 mM, was tested. Control samples did not contain glucose. Glucose, hydrogen, volatile fatty acids (VFA) and ethanol concentrations were measured at the beginning and at the end of incubation time (5 days). Experiments were done in 117.5 mL serum bottles with 50 mL liquid medium. All experiments were done in duplicate and the pH was analysed in the end of incubation time in all cases.

Substrate utilization

The ability of strain AK_{17} to utilize different substrates was tested using the BM medium supplemented with various filter sterilized substrates (20 mM or 2 g L^{-1}). Substrates tested were: xylose, ribose, arabinose, glucose, fructose, galactose, mannose, sucrose, lactose, lactate, formate, succinate, malate, pyruvate, oxalate, crotonate, glycerol, inositol, starch, cellulose, xylan, sorbitol, pectin, casamino acids, peptone, beef extract, tryptone, alanine, aspartate, glycine, glutamate, serine, theronine, histidine and cysteine. Optical density was measured at the beginning and at the end of incubation time (5 days). Where growth was detected, hydrogen, volatile fatty acids and ethanol were analyzed. Experiments were done in 24.5 mL serum bottles with 10 mL liquid medium.

Pretreatment of biomass and hydrolysates preparation

Hydrolysates (HL) were made from different biomass: Whatman filter paper (cellulose – control sample), hemp (*Cannabis sativa*) – leafs and stem fibres, newspaper with ink (NPi), barley straw (BS) (*Hordeum vulgare*) and grass (*Phleum pratense*). HL's were prepared according to Sveinsdottir *et al*. (2009), yielding a final dry weight of 25 g L^{-1}. Biomass was in some cases pretreated chemically by using different concentrations (0.25 to 2.00%) of acid (H_2SO_4) or alkali (NaOH) (control was without chemical pretreatment) before autoclaving for 60 minutes (121°C). Enzymes, Celluclast® and Novozyme 188,

were added to each bottle after chemical pretreatment; the bottles were cooled down to room temperature and the pH adjusted to 5.0 before enzymes were added. In most experiments, 0.1 mL g^{-1} (dw) of each enzyme was added to the hydrolysates which were then incubated in water bath at 45°C for 68h. In one experimental set up, different concentrations of enzymes were used, i.e. 0.01-0.20 mL g^{-1} (dw). After the enzyme treatment the pH was measured again and adjusted to pH 6.0 which is the pH optimum of the strain. The hydrolysates were then filtered (Whatman – WeiBrand; 0.45 µm) into sterile bottles.

Fermentation of hydrolysates

Fermentation of carbohydrates present in hydrolysates was performed in 24.5 mL serum bottles. The medium (8 mL) was supplemented with hydrolysates (2 mL – total liquid volume of 10 mL) giving a final hydrolysate concentration of 5.0 g L^{-1}. Control samples did not contain hydrolysate. In one experimental set up, different concentrations of hydrolysates were added. Concentrations of hydrolysates varied from 2.5 g L^{-1} to 17.5 g L^{-1}.

Effect of toxic compounds on ethanol production

The effect of furfural and hydroxymethylfurfural (HMF) on ethanol production by AK_{17} was tested. BM media was supplemented with glucose (20 mM) and either furfural or HMF in concentration varying from 0.0 – 6.0 g L^{-1}. Glucose, hydrogen, volatile fatty acids (VFA) and ethanol concentrations were measured at the beginning and at the end of incubation time (5 days). Experiments were done in 117.5 mL bottles with 50 mL liquid medium.

Analytical methods

Hydrogen, ethanol and volatile fatty acids were measured by gas chromatograph as previously described (Orlygsson & Baldursson, 2007). Glucose was determined by slight modification of the method from Laurentin & Edwards (2003); liquid sample (400 µL) was mixed with 2 mL of anthrone solution (0.2% anthrone in 72% sulfuric acid). The sample was boiled for 11 minutes and then cooled down on ice. Optical density was then measured at 640 nm by using Perkin Elmer spectrophotometer (Lambda 25).

Results and discussion

Characteristics of strain AK_{17}

Previous 16S rRNA analysis of strain AK_{17} (DSM 18780) has revealed that it belongs to the genus *Thermoanaerobacterium* (Orlygsson and Baldursson, 2007; Sveinsdottir et al. 2009), with *T. aciditolerans* being its closest relative (99.1% homology). The strain has a temperature and pH optimum at 58°C and 6.0, respectively, with a generation time around 1.0 h. It has a slightly bended rod shape (Fig. 1a and b) and stains Gram positive.

As revealed by the degradation spectrum of glucose and xylose, strain AK_{17} is a very good ethanol producer. Degradation of 20 mM glucose and xylose resulted in 29.9 and 21.3 mM of ethanol (Sveinsdottir *et al*., 2009) which corresponds to 1.5 and 1.1 mol EtOH mol $glucose^{-1}$ and $xylose^{-1}$, respectively (= 8.3 and 7.1 mM/g glucose and xylose, respectively). These yields are among the highest reported by thermophilic bacteria. *Thermoanaerobacter ethanolicus* produces 1.9 mol-EtOH mol-$glucose^{-1}$ in batch (Wiegel & Ljungdahl, 1981) and continuous cultures (Lacis & Lawford, 1991). Fermentation of xylose by *T. ethanolicus* results in 1.37 mol-EtOH (Lacis and Lawford, 1991). *Thermoanaerobacter thermohydrosulfuricum* has also been reported to produce 1.5 mol-EtOH mol-$glucose^{-1}$ (Wiegel *et al*., 1979).

The strain also produces acetate, hydrogen and carbon dioxide. The stoichiometric degradation of glucose and xylose resulted in the following equations.

20 mM Glucose → 29.9 mM EtOH + 7.5 mM Acetate + 13.8 mM H_2 + 41.7 mM CO_2

20 mM Xylose → 21.3 mM EtOH + 8.3 mM Acetate + 14.3 mM H_2 + 29.6 mM CO_2

The substrate spectrum of the strain has also been investigated (Orlygsson and Baldursson, 2007) but volatile end product formation has not been presented earlier. The strain degrades glucose, fructose, mannose, galactose, xylose, arabinose, ribose, lactose, sucrose, xylan, pectin and pyruvate and, in all cases, produces acetate, ethanol, hydrogen and carbon dioxide (Fig. 2). Among substrates that were not utilized were amino acids and organic acids. This substrate spectrum is similar to most of other known *Thermoanaerobacterium* species (Collins *et al*., 1994; Liu *et al*., 1996; Kublanov *et al*., 2007), namely being highly saccharolytic but not able to degrade cellulose.

Effect of increased substrate loading on ethanol production

High initial substrate concentration may inhibit substrate utilization and/or decrease end product yields (Lacis & Lawford 1988; van Ginkel & Sung, 2001; Sommer *et al*., 2004). In closed systems, such as batch cultures, the limited buffer capacity of the medium may be over loaded by the accumulation of organic acids resulting in a pH drop and the inhibition of substrate fermentation utilization (van Ginkel & Logan, 2005; Olsson & Hahn-Hägerdal, 1996). In present study, increased glucose loadings led to lower pH's, increased acetate formation and a lower degree of glucose utilized in the cultures (Table 1). By increasing the initial glucose concentration from 5 mM to 10 mM, a twofold increase in end product formation was observed and more than 98% of the glucose was degraded in both cases. However, at 30 mM glucose concentration, only 80% of the glucose was degraded and end product formation did not increase proportionally to increased substrate loadings. At higher loadings (≥ 50mM) no further increase in end products were observed and only a small fraction of the glucose was degraded. The pH was measured after fermentation and decreased from low glucose loadings (pH 6.1) to high (≥ 50mM) loadings (pH 5.2). This, together with the fact that end product formation levels off at high substrate loadings indicate that this inhibition is more likely to be caused by the low pH rather than the high substrate loadings.

Fermentation of hydrolysates from lignocellulosic biomass

The strain is producing more than 40 mM of ethanol from hydrolysates made from cellulose (Table 2). The yields on cellulose are similar (8.2 mM g^{-1} dw) as compared to glucose degradation alone (eq. 1) indicating a 100% hydrolysis of cellulose to glucose. Other end products (acetate, hydrogen) were also produced in the same proportions as observed on glucose alone. Not surprisingly, addition of acid or alkali agents did not increase end product formation yields on cellulose as was clearly observed on lignocellulosic substrates. Highest ethanol yields on the more complex biomass types (without chemical pretreatment) were observed on hemp stem (2.1 mM g^{-1} dw) but lowest on hemp leaves (0.4 mM g^{-1} dw). Addition of either acid or alkali increased yields substantially on all lignocellulosic biomass tested. The increase was most profound on hydrolysates from hemp leaves (2.63 times) and straw (2.52 times), both pretreated with acid.

Earlier experiments on the capacity of strain AK_{17} to produce ethanol from hydrolysates made from lignocellulosic biomass have shown that the strain produced 5.8 mM ethanol g^{-1} cellulose at a concentration of 7.5 g L^{-1} (Sveinsdottir *et al*., 2009). The reason for higher yields in present investigation are most likely due to lower hydrolysate concentration used (5 g L^{-1}). Various data available on ethanol production from lignocellulosic biomass in both pure cultures and mixed cultures are presented in Table 3. The highest ethanol yields are reported by *Thermoanaerobacter* strain BG1L1 on wheat straw (Georgieva *et al*., 2008) and corn stover (Georgieva & Ahring, 2007), which corresponds to 8.5 - 9.2 mM g^{-1} sugar consumed. Among other studies with good yields from lignocellulose are e.g. *Clostridium thermocellum* on paddy straw, sorghum stover and corn stubs (Rani *et al*., 1997) and *Thermoanaerobacterium saccharolyticum* on xylan (Ahring *et al*., 1996).

Effects of different concentrations of grass and cellulose hydrolysates

In order to investigate further the capacity of the strain to produce ethanol from lignocellulosic biomass it was decided to use grass hydrolysates and cellulose (as control) without chemical pretreatment at different concentrations (5.0 to 17.5 g L^{-1}). Increased concentrations of cellulose hydrolysates inhibit end product formation by strain AK_{17} (Table 4) further supporting that high initial glucose concentrations are of great importance for the bacterium. At lowest cellulose concentrations used, the ethanol yield was 7.5 mM g^{-1} but drops to only 2.4 mM g^{-1} at 17.5 g L^{-1}. On grass hydrolysates a steady increase in end product formation was observed by increasing HL loadings and at highest concentrations ethanol yields were similar (1.3 to 1.4 mM g^{-1} hydrolysate) compared to lower HL loadings, indicating no inhibition in end product formation. The most reasonable explanation for this difference between grass and cellulose HL's is that the glucose content after enzyme pretreatment is considerably lower in grass hydrolysate because of various other sugars present in the hemicellulose fraction (not analyzed). Additionally, acetate concentrations were higher in the cellulose hydrolysates resulting in a pH drop at end of incubation and could partly explain lower ethanol yields.

Effects of different concentrations of NaOH and H_2SO_4 hydrolysates

Dilute-acid processes have been viewed primarily as a means of pre-treatment for the hydrolysis of hemicelluloses leaving the cellulose fraction more accessible for a further enzymatic treatment (Mosier *et al*., 2005). Typical sulphuric acid concentrations for

hemicellulose hydrolysis are in the range of 0.5–1.5%. The use of alkaline based metals (sodium, potassium and calcium) for pretreatment is mostly directed towards lignin solubilisation exhibiting only minor cellulose and slightly higher hemicellulose solubilisation.

In order to investigate the effects of chemical pretreatment on ethanol yields from both cellulose and grass HL, different concentrations of both H_2SO_4 and NaOH were used. Not surprisingly, little difference in ethanol yields were observed from cellulose HL by using either acid or alkali at any of the concentrations used (Table 5). The ethanol yields from grass HL without chemical pretreatment resulted in 11.3 mM (1.9 mM g^{-1}). Chemical pretreatment with either acid or alkali in low concentrations (0.25%), increased yields to 3.2 and 3.5 mM g^{-1}), respectively. Further increase in acid concentration led to slightly increased ethanol yields whereas addition of more alkali did not increase ethanol yields. Thus, relatively low acid/alkali concentrations may be used for pretreatment of grass HL for good ethanol yields. It is likely however, that higher yields may be obtained by using e.g. two step temperature processes for the dilute acid pretreatment (Gírio *et al.*, 2010).

Effects of different enzyme concentrations

One of the major bottlenecks for ethanol production from lignocelluloses in large scale is the high enzyme cost (Bevill, 2009). In all experiments presented in this study, the amount of Celluclast® and Novozyme 188 (each) were 0.1 mL g^{-1} (dw) of hydrolysates. To investigate the effect of different amount of enzymes added to hydrolysates the enzyme volume was stepwise reduced to 0.01 mL g^{-1} dw as well as increasing it twofold. No end product formation was observed without enzyme addition from cellulose hydrolysates. By adding only one tenth of the amount used earlier, a dramatic increase (from zero to 3.5 mM g^{-1}) was observed in ethanol yields (Table 6). Ethanol yields were further increased by increasing enzyme concentration up to 9.7 mM g^{-1}. Surprisingly; the yields of ethanol from grass hydrolysates were 0.9 mM/g without adding any enzyme; this was, however, increased to 1.9 mM g^{-1} at 0.1 mL enzyme concentrations.

Effects of furfural and hydroxymethylfurfural on growth

Furfural and hydroxymethylfurfural (HMF) are furan derivatives from pentoses and hexoses, respectively. These two compounds have been reported to act as strong inhibitors of glycolytic enzymes (Palmquist *et al*., 2000; Banaerjee *et al*., 1981).

In this study, the inhibitory effects of both of these compounds were observed. At 0.5 g L^{-1} of furfural or HMF, the decrease in ethanol production was 17 and 15%, respectively (Fig. 2). For furfural this decrease in ethanol was approximately 50% at 2 g L^{-1} but no growth was observed at higher concentrations. For HMF, slightly less decrease was observed with increasing HMF concentrations and the minimum inhibitory concentration was higher (4 g L^{-1}). The strain is less sensitive to furan compounds as compared to *Thermoanaerobacterium saccharolyticum* (Cao *et al*., 2010) but similar as *Thermotoga neopolitana* (de Vrije *et al*., 2009). This inhibition should be taken into consideration when applying hydrolysates from lignocellulosic biomass, either by using low HL concentrations or by reducing furan derivatives.

Optimization experiment

From the data obtained from the various experiments on grass and cellulose HL it was decided to make an optimization experiment where the following parameters were used: concentration of acid/alkaline, 0.25%; enzyme concentration, 0.1 g L^{-1}; concentration of hydrolysates, 2.5, 5.0 and 7.5 g L^{-1}. Clearly, the most important factor for ethanol yields is the concentrations of the HL used (Fig. 3). By lowering the HL concentration from 7.5 to 5.0 and further to 2.5 g L^{-1} the ethanol yields increase from 5.8, 7.5 and 8.6 mM g^{-1} cellulose (Fig. 3a) and 4.8, 4.9 and 5.5 mM g^{-1} grass hydrolysates (Fig. 3b), respectively. Increased loadings also led to increased acetate and hydrogen production and lower pH at the end of fermentation time. Thus, the pH for cellulose HL decreased from 5.9 at 2.5 g/L to 4.3 at 7.5 g/L, respectively. Less decrease was observed for the grass HL (6.2 to 5.5) because of the lower amounts of acetate produced.

Conclusion

In this study, bioethanol production in *Thermoanaerobacterium* AK_{17} was characterized and optimized. The aciditolerant bacterium is capable of producing ethanol with a yield as high as 1.5 mol-ethanol mol-glucose^{-1} and 1.1 mol-ethanol mol xylose^{-1}. Optimization experiments on grass and cellulose hydrolysates resulted in very high ethanol yields or 5.5 and 8.6 mM g^{-1}, respectively. End product formation was clearly inhibited by addition of furfural and hydroxymethylfurfural.

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Tables

Table 1. End product formation from different initial glucose concentrations. Also shown are residual glucose concentrations after fermentation and end pH values. Values are the mean of two replicates.

Initial glucose (mM)	End product formation (mmol L^{-1})			End glucose (mM)	End pH
	Ethanol	Acetate	Hydrogen		
0	1.8	2.0	1.1	0.0	6.0
5	9.8	4.2	6.1	0.3	6.1
10	16.0	8.4	13.1	0.4	5.7
30	34.4	14.5	24.5	6.0	5.1
50	29.9	10.2	21.2	24.9	5.2
100	38.2	9.3	18.5	72.1	5.2
200	32.1	7.8	13.2	159.5	5.2

Table 2. Production of end products from hydrolysates (5 g L^{-1}) from different biomasses. Values represent the mean of two replicates. Ethanol yields in mM g^{-1} dw are given within brackets.

	End product formation (mmol L^{-1})		
Biomass and pretreatment	Ethanol	Acetate	Hydrogen
Control	1.8 (N.d)	2.0 (N.d)	1.1 (N.d)
Cellulose	43.0 (8.2)	15.3 (2.7)	34.0 (6.6)
Cellulose, acid	42.6 (8.2)	14.5 (2.5)	29.7 (5.7)
Cellulose alkali	42.8 (8.2)	14.6 (2.5)	32.8 (6.3)
Hemp stem	12.2 (2.1)	6.8 (1.0)	14.6 (2.7)
Hemp stem, acid	14.1 (2.5)	9.6 (1.5)	15.1 (2.8)
Hem stem, alkali	14.6 (2.6)	10.4 (1.7)	15.5 (2.9)
Hemp leaf	3.6 (0.4)	3.2 (0.2)	3.8 (0.5)
Hemp leaf, acid	9.5 (1.5)	6.0 (0.8)	11.2 (2.0)
Hemp leaf, alkali	5.5 (0.7)	4.9 (0.6)	7.9 (1.4)
Grass	9.5 (1.5)	4.4 (0.5)	9.4 (1.7)
Grass, acid	20.0 (3.6)	7.0 (1.0)	13.5 (2.5)
Grass, alkali	18.6 (3.4)	8.8 (1.4)	18.0 (3.4)
Paper	4.7 (0.6)	2.6 (0.1)	4.2 (0.6)
Paper, acid	9.5 (1.5)	6.0 (0.8)	11.0 (2.0)
Paper, alkali	5.9 (0.8)	4.5 (0.5)	7.1 (1.2)
Straw	6.4 (0.9)	3.6 (0.3)	7.1 (1.2)
Straw, acid	16.1 (2.9)	8.1 (1.2)	13.4 (2.5)
Straw, alkali	10.8 (1.8)	6.6 (0.9)	9.2 (1.6)

N.d = not determined

Table 3. Ethanol yields from different biomasses – selected data from literature.

Organism	Biomass	Substrate concentration (g/L)	Pretreatment	EtOH yield (mM g^{-1})	References
Clostridium thermocellum	Whatman paper	8,0	None	7.2-8.0	Rani et al. (1997)
Clostridium thermocellum	Avicel	8,0	None	6.5-7.2	Rani et al. (1997)
Clostridium thermocellum	Paddy straw	8,0	Alkali	6.1-8.0	Rani et al. (1997)
Clostridium thermocellum	Sorghum stover	8,0	Alkali	4.8-8.1	Rani et al. (1997)
Clostridium thermocellum	Corn stubs	8,0	Alkali	4.6-7.8	Rani et al. (1997)
Thermoanaerobacter mathranii	Wheat straw	6,7	Wet oxidation	1.34	Ahring et al. (1999)
Thermoanaerobacter BG1L1	Corn stover	25.0-150.0	Acid	8.5-9.2	Georgieva & Ahring (2007)
Thermoanaerobacter BG1L1	Wheat straw	30.0-120.0	Wet oxidation	8.5-9.2	Georgieva et al. (2008)
Thermoanaerobacter ethanolicus	Been card	10.0	Dryed, crushed	1.8	Miyazaki et al. (2008)
Thermoanaerobacterium AK17	Whatman paper	7,5	Enzymes	5,81	Sveinsdottir et al. (2009)
Thermoanaerobacterium AK17	Grass	7,5	Enzymes	2,91	Sveinsdottir et al. (2009)
Thermoanaerobacterium AK17	Paper	7,5	Enzymes	2,03	Sommer et al. (2004)
Thermoanaerobacterium AK17	Whatman paper	2,5	Acid/alkali, enzymes	8.60	This study
Thermoanaerobacterium AK17	Grass	2,5	Acid/alkali, enzymes	5.50	This study

Table 4. End product formation from different initial cellulose and grass hydrolysate concentrations. Values represent the mean of two replicates. Ethanol yields in mM g^{-1} dw are given within brackets.

Hydrolysate concentrations ($g\ L^{-1}$)	End product formation ($mmol\ L^{-1}$)			
	Ethanol	Acetate	Hydrogen	End pH
Control	1.8 (N.d)	2.0 (N.d)	1.1 (N.d)	6.0
Cellulose, 5.0	47.1 (9.5)	10.2 (2.0)	23.6 (4.7)	5.5
Cellulose, 7.5	43.4 (5.8)	10.1 (1.3)	32.7 (3.4)	5.5
Cellulose, 10.0	47.2 (4.7)	15.8 (1.6)	29.4 (2.9)	4.9
Cellulose, 12.5	36.6 (2.9)	13.8 (1.1)	27.9 (2.2)	5.0
Cellulose, 15.0	35.4 (2.4)	13.5 (0.9)	27.9 (1.9)	5.0
Cellulose, 17.5	37,2 (2.1)	12.6 (0.7)	26.3 (1.5)	5.1
Grass, 5.0	8.9 (1.4)	6.0 (1.2)	5.5 (0.9)	5.7
Grass, 7.5	12.4 (1.4)	6.9 (0.9)	6.8 (0.8)	5.8
Grass, 10.0	15.9 (1.4)	8.6 (0.9)	8.4 (0.7)	5.6
Grass, 12.5	17.7 (1.3)	9.5 (0.8)	10.4 (0.7)	5.5
Grass, 15.0	20.6 (1.3)	11.4 (0.8)	12.4 (0.8)	5.4
Grass, 17.5	23.8 (1.3)	12.6 (0.7)	13.4 (0.7)	5.3

Nd = not determined

Table 5. End product formation from cellulose and grass hydrolysates (5 g L^{-1}) pretreated with different concentrations of H_2SO_4 and NaOH. Values represent the mean of two replicates. Ethanol yields in mM g^{-1} dw are given within brackets.

Biomass and pretreatment	End product formation (mmol L^{-1})			
	Ethanol	Acetate	Hydrogen	End pH
Control without biomass	1.8	2.0	1.1	6.2
Cellulose, 0.00% acid/alkali	41.1 (7.9)	13.1 (2.2)	23.5 (4.5)	5.3
Cellulose, 0.25% acid	40.3 (7.7)	12.1 (2.0)	19.4 (3.7)	5.5
Cellulose, 0.50% acid	38.7 (7.4)	11.5 (1.9)	18.4 (3.5)	5.4
Cellulose, 0.75% acid	42.3 (8.1)	10.8 (1.8)	21.1 (4.0)	5.7
Cellulose, 1.00% acid	41.4 (7.9)	11.9 (2.0)	20.8 (3.9)	5.4
Cellulose, 2.00% acid	38.4 (7.3)	10.1 (1.6)	19.6 (3.7)	5.6
Cellulose, 0.25% alkali	34.6 (6.6)	10.5 (1.7)	19.4 (3.7)	5.4
Cellulose, 0.50% alkali	40.9 (7.8)	12.1 (2.0)	20.3 (3.8)	5.2
Cellulose, 0.75% alkali	35.9 (6.8)	10.1 (1.6)	16.9 (3.2)	5.2
Cellulose, 1.00% alkali	42.9 (8.2)	10.6 (1.7)	19.4 (3.7)	5.2
Cellulose, 2.00% alkali	30.9 (5.8)	8.6 (1.3)	12.8 (2.3)	5.3
Grass, 0.00% acid/alkali	11.3 (1.9)	4.8 (0.6)	6.0 (1.0)	6.1
Grass, 0.25% acid	17.8 (3.2)	7.7 (1.1)	8.4 (1.5)	5.7
Grass, 0.50% acid	20.1 (3.7)	8.5 (1.3)	8.6 (1.5)	5.7
Grass, 0.75% acid	19.7 (3.6)	8.6 (1.3)	9.7 (1.7)	5.6
Grass, 1.00% acid	19.9 (3.6)	8.2 (1.2)	8.6 (1.5)	5.6
Grass, 2.00% acid	20.1 (3.7)	7.6 (1.1)	7.5 (1.3)	5.8
Grass, 0.25% alkali	19.1 (3.5)	8.0 (1.2)	7.2 (1.2)	5.8
Grass, 0.50% alkali	18.8 (3.4)	8.2 (1.2)	7.9 (1.4)	5.7
Grass, 0.75% alkali	17.8 (3.2)	8.2 (1.2)	8.9 (1.6)	5.7
Grass, 1.00% alkali	17.7 (3.2)	8.1 (1.2)	7.9 (1.4)	5.7
Grass, 2.00% alkali	15.7 (2.8)	7.2 (1.0)	7.8 (1.4)	5.7

N.d = not determined

Table 6. End product formation from cellulose and grass hydrolysates (5 g L^{-1}) pretreated with different concentrations of enzymes (Novozyme 188 and Celluclast®). Values represent the mean of two replicates. Ethanol yields in mM g^{-1} dw are given within brackets.

Biomass and enzyme concentration (ml g^{-1} dw)	End product formation (mmol L^{-1})			End pH
	Ethanol	Acetate	Hydrogen	
Control	1.8 (N.d)	2.0 (N.d)	1.1 (N.d)	6.3
Cellulose (0.0)	2.1 (0.1)	2.0 (0.0)	1.4 (0.1)	6.4
Cellulose (0.01)	19.2 (3.5)	6.1 (0.8)	11.3 (2.0)	5.9
Cellulose (0.02)	27.1 (5.1)	7.7 (1.1)	12.8 (2.3)	5.9
Cellulose (0.04)	38.4 (7.3)	9.8 (1.6)	15.1 (2.8)	5.5
Cellulose (0.06)	40.8 (7.8)	9.9 (1.6)	15.8 (2.9)	5.5
Cellulose (0.08)	50.1 (9.7)	14.5 (2.5)	24.6 (4.7)	4.9
Cellulose (0.10)	49.9 (9.6)	12.9 (2.2)	22.5 (4.4)	5.2
Cellulose (0.20)	52.2 (10.2)	13.8 (2.4)	24.5 (4.7)	5.0
Grass (0.0)	6.2 (0.9)	3.0 (0.2)	3.9 (0.6)	6.2
Grass (0.01)	8.2 (1.3)	3.2 (0.2)	4.6 (0.7)	6.1
Grass (0.02)	9.1 (1.5)	3.1 (0.2)	4.6 (0.7)	6.1
Grass (0.04)	8.1 (1.3)	3.1 (0.2)	3.2 (0.4)	6.2
Grass (0.06)	10.4 (1.7)	2.9 (0.2)	3.8 (0.6)	6.1
Grass (0.08)	11.2 (1.9)	2.8 (0.2)	3.7 (0.5)	6.2
Grass (0.10)	8.3 (1.3)	3.2 (0.2)	3.5 (0.5)	6.1
Grass (0.20)	8.8 (1.4)	3.2 (0.2)	3.3 (0.4)	6.2

N.d = not determined

Figure 1: Phase contrast and electronic microscopy picture of strain AK_{17}.

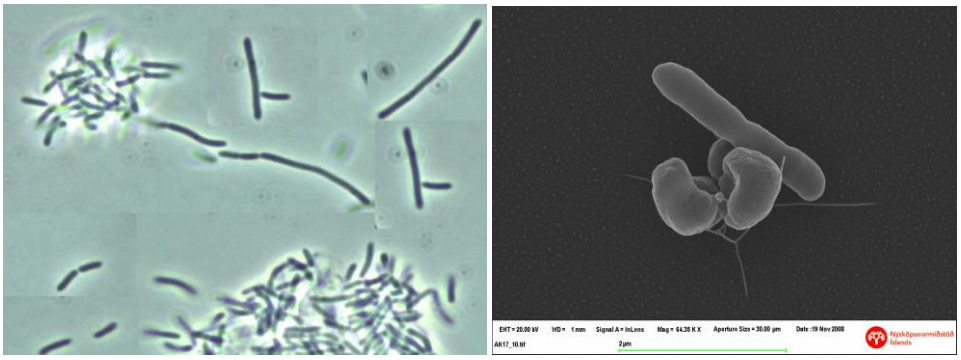


Figure 2: End product formation from various substrates by strain AK_{17}. Values represent mean of two replicates.

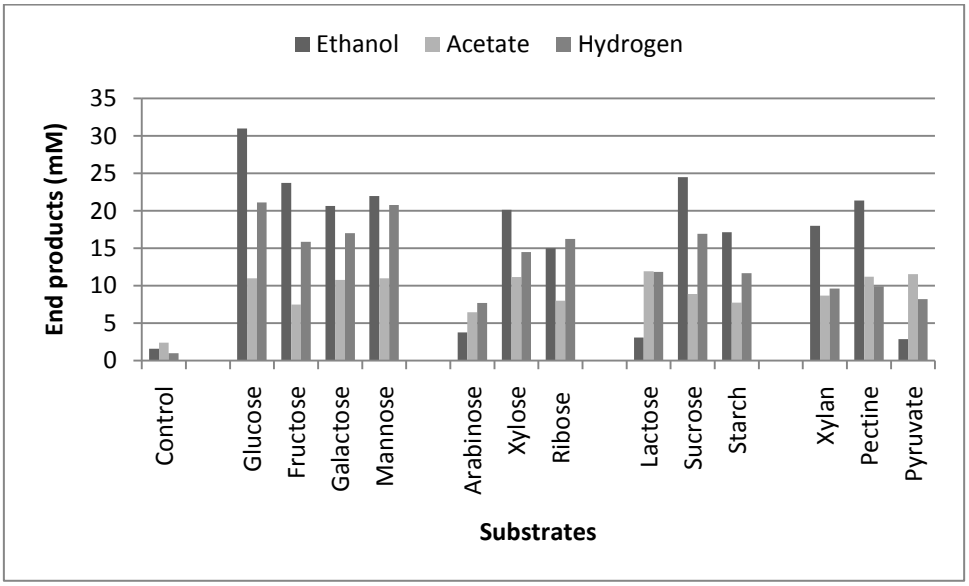
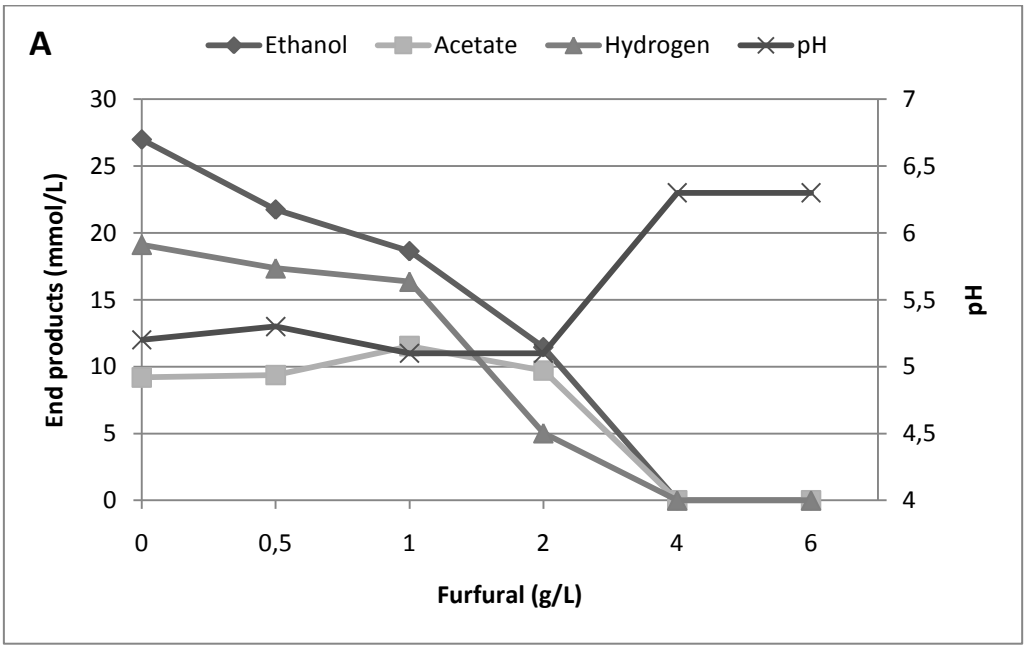


Figure 3: End product formation from glucose (20 mM) in the presence of different concentrations of furfural (A) and hydroxymethylfurfural (B) by strain AK_{17}.



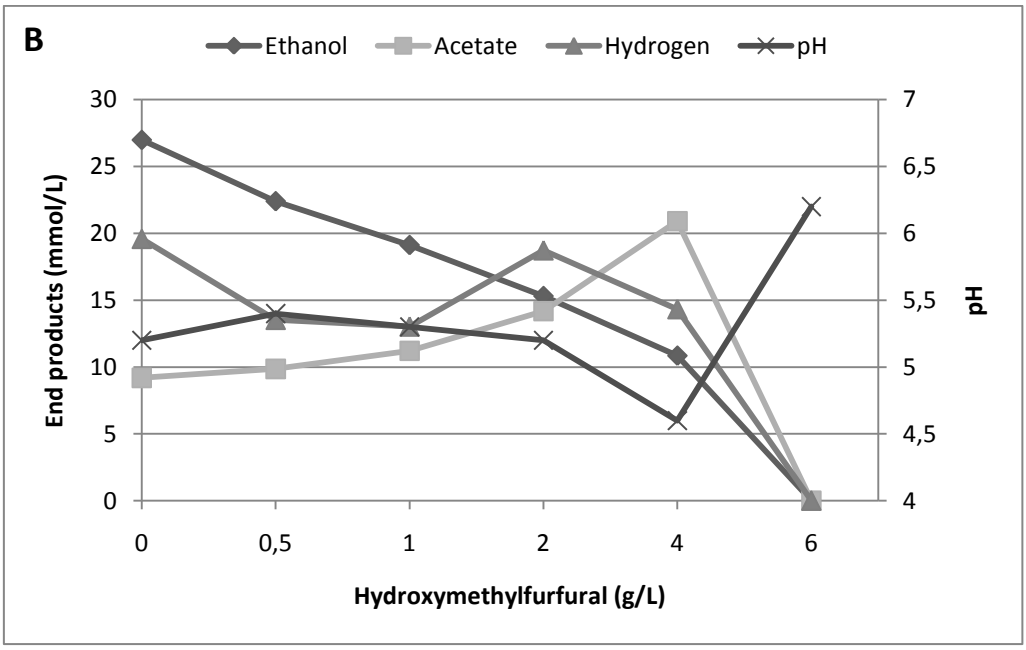
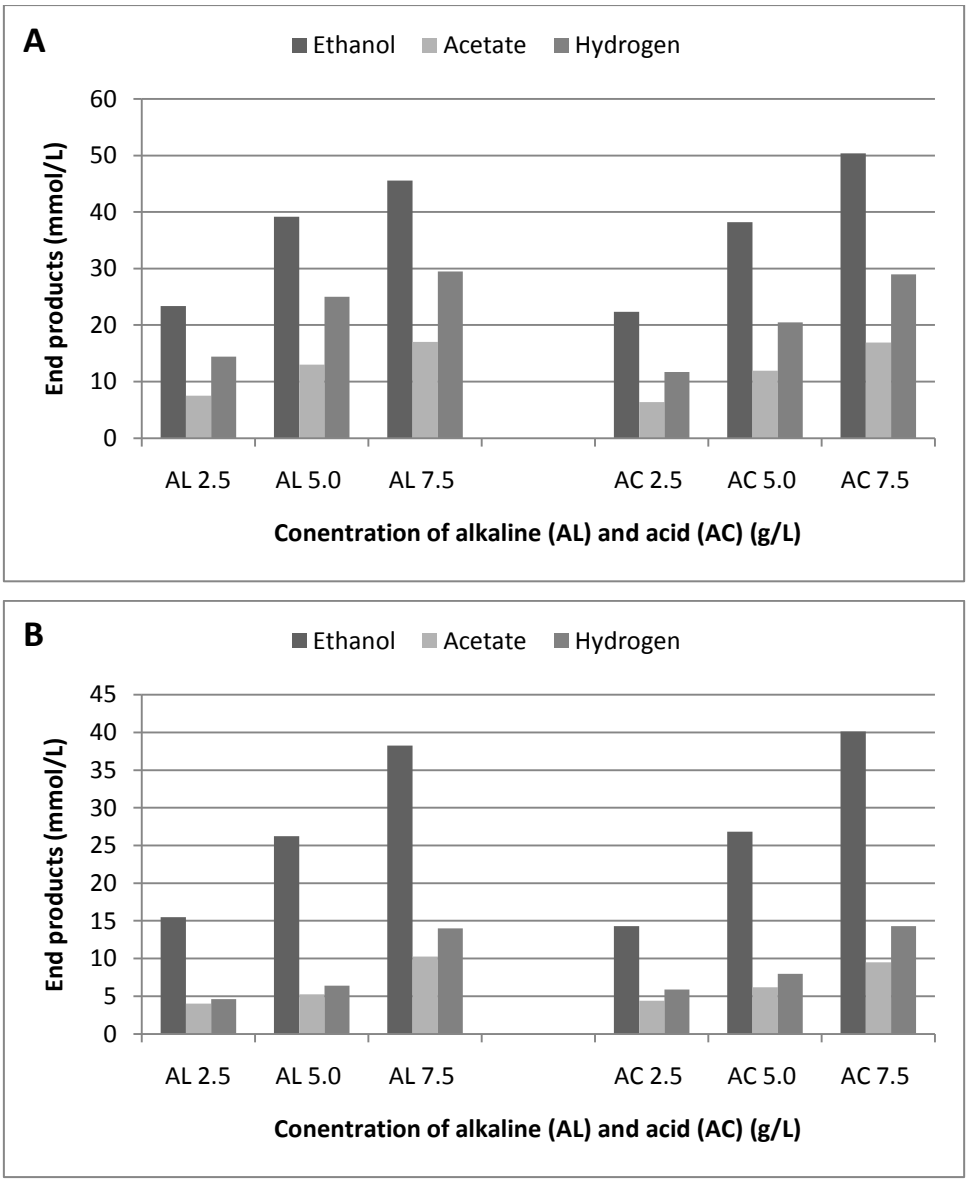


Figure 4: Optimization of end product formation from cellulose (A) and grass hydrolysates (B). Concentrations of hydrolysates used were 2.5, 5.0 and 7.5 g L^{-1}, enzyme concentrations 0.1 ml g $biomass^{-1}$, and the concentration of H_2SO_4 and NaOH during pretreatment was 0.25%



7. Conclusion

Fermentative thermophilic bacteria have gained increased attention in the last few years because of their wide substrate utilization spectra, fast growth rates and good ethanol and hydrogen yields.

The present study is examines two thermophilic bacteria that have been shown to have good hydrogen and ethanol production capacity; both utilize various sugars as well as hydrolysates made from lignocellulosic biomass. Various physiological experiments were performed on *Clostridium* strain AK_{14} to examine optimal growth conditions, the effect of initial substrate concentration, substrate utilization spectrum, - and the kinetics of glucose degradation and end product formation. The fermentation spectrum of this strain shows the classical acetate-butyrate fermentation with high hydrogen yields. During batch growth on 20 mM of glucose and xylose the bacteria produced 30.5 and 35.3 mmol H_2 L^{-1}. Growth on hydrolysates made from cellulose (Whatman paper) resulted 8.5 mol H_2 g^{-1} VS but when grown on more complex biomass like news paper and lignocellulosic biomass resulted in lower yields (0.26 to 3.60 mol H_2 g^{-1} VS). Alkali and acid pretreatments of the complex biomass increased H_2 yields substantially. The partial pressure of hydrogen (pH_2) was also shown to be of great importance for end product formation; at high pH_2 more reduced end products (e.g. ethanol) were produced and hydrogen yields were lowered from 2.6 to 1.6 mol H_2/mol glucose.

The ethanol production capacity from sugars and complex biomass by *Thermoanaerobacterium* strain AK_{17} was also studied. The strain degrades various carbohydrates and the primary end products are ethanol, acetate, hydrogen and CO_2. Batch culture studies on glucose and xylose resulted in 1.5 and 1.1 mol ethanol/mol sugars, respectively. Ethanol production from various hydrolysates made from cellulose and lignocellulosic biomass showed good ethanol yields. Fermentation of cellulose and grass hydrolysates resulted in 8.2 and 1.5 mM ethanol g^{-1} substrate, respectively. The use of alkali or acid as pretreatment enhanced ethanol yields from lignocellulosic biomass substantially, e.g. from 1.5 to 3.6 mM g^{-1} grass.

The influence of various environmental factors (concentrations of hydrolysates, enzymes and acid/alkaline) on end-product formation from lignocellulosic hydrolysates as well as the effect of inhibitory compounds were investigated in detail. Optimization experiments for ethanol production at low hydrolysate concentrations (2.5 g L^{-1}); with 0.25% acid/alkali (v/v) and 0.1 mL g^{-1} enzyme concentrations resulted in 5.5 and 8.6 mM

g^{-1} grass and cellulose, respectively. Inhibitory effects of furfural and hydroxymethylfurfural, revealed a total inhibition of end product formation from glucose at 4 and 6 g/L, respectively.