Master thesis for 90 credit M.Sc. in Biotechnology

Thermoanaerobacter:

Potential ethanol and hydrogen producers

Hrönn Brynjarsdóttir

Supervisor: Jóhann Örlygsson

University of Akureyri
School of business and science
Faculty of natural resources
May 2012

Master thesis for 90 credit M.Sc. in Biotechnology

Thermoanaerobacter

Potential ethanol and hydrogen producers

Hrönn Brynjarsdóttir

Supervisor: Jóhann Örlygsson

University of Akureyri
School of business and science
Faculty of natural resources
May 2012

Declaration

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

_____

Hrönn Brynjarsdóttir

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 and Science.

_____

Dr. Jóhann Örlygsson

Abstract

Two anaerobic, thermophilic bacterial strains, isolated from hot springs in Grensdalur, SW-Iceland, were examined concerning their ability to produce biofuels (ethanol and hydrogen) from various sugars and hydrolysates of complex biomass. Fifteen isolates from the strain collection of the University of Akureyri were originally screened for ethanol and hydrogen production capacity on glucose, xylose and hydrolysates from Whatman paper (cellulose) and grass. Based on results of these cultivations one strain (AK_5) was selected because of high ethanol production capacity and another strain (CMC_{15}) with high hydrogen production capacity. A partial 16S rRNA analysis revealed that both strains belong to the genera *Thermoanaerobacter*. Hydrogen and ethanol production from various carbon sources were investigated as well as the effects of various environmental factors (substrate concentration, effect of pH, temperature and partial pressure of hydrogen) on growth and end product formation. The effects of external electron scavenging systems on end product formation was also investigated for strain AK_5. Finally, biofuel production from hydrolysates made from various lignocellulosic biomass was investigated in detail and compared with literature values. Maximum ethanol production of strain AK_5 was 1.7 mol/mol of glucose; 1.35 mol/mol xylose; 7.7 mmol/g cellulose og 4.4 mmol/g grass. During growth in the presence of electron scavenging systems the strain almost completely shifted from ethanol to acetate formation. The main flaw of the strain was its intolerance towards high glucose concentrations but this may be minimized by using fed-batch or continuous culture techniques. Strain CMC_{15} maximally produced 3,1 mol H_2/mol glucose and 4.83 mol H_2/g grass hydrolysate. Similar to strain AK_5 this strain was also inhibited by increasing glucose concentrations, most likely because of acetate production which results in low pH during batch culture cultivation. Thus, continuous culture with stable pH regulation should be used for scaling up the cultivation of the strain.

Keywords: *Thermoanaerobacter*, thermophilic, ethanol, hydrogen, complex biomass

Acknowledgements

I would like to thank my mentor, Dr. Jóhann Örlygsson for the opportunity to work on this interesting project and his guidance and support. I would like to thank my coworkers, friends and helpers at the lab: Margrét Auður Sigurbjörnsdóttir, Arnheiður Rán Almarsdóttir, Beata Wawiernia, Alicja Tarazewicz, Jan Eric Jessen and Árný Ingveldur Brynjarsdóttir.

I would also like to thank my husband, my two sons and my parents for all their patience and support through my two years of research.

Thanks to Orkurannsóknasjóður Landsvirkjunar for choosing me as one of the masterstudents they supported with a grant in 2011.

Special thanks to Sean M. Scully and my husband, Pálmi Óskarsson for proofreading.

Thank you all

Hrönn Brynjarsdóttir

25.04.2012

Útdráttur

Tveir loftfirrtir, hitakærir bakteríustofnar, einangraðir úr heitum hverum í Grensdal við Hveragerði, voru rannsakaðir með tilliti til getu þeirra til að framleiða lífeldsneyti (etanól og vetni) úr mismunandi sykrum og úr hýdrólýsötum úr flóknum lífmassa. Fimmtán stofnar úr stofnasafni Háskólans á Akureyri voru ræktaðir á glúkósa, xýlósa og hýdrólýsötum úr whatman pappír (sellulósa) og grasi. Af þessum stofnum var einn etanólframleiðandi stofn (AK_5) og einn vetnisframleiðandi stofn (CMC_{15}) valdir til frekari rannsókna. Hlutraðgreining (16S rRNA) á stofnunum sýnir að báðir tilheyra ættkvíslinni *Thermoanaerobacter*. Stofn AK_5 er skyldastur *T. thermohydrosulfuricus* (99,1% skyldleiki) og stofn CMC_{15} er *T. yonseiensis* (98,9% skyldleiki). Margvíslegar tilraunir voru gerðar til að kanna getu bakteríanna til að brjóta niður fjölmarga kolefnagjafa auk þess sem könnuð voru áhrif margra umhverfisþátta (hitastig, pH, styrkur hvarfefna, hlutþrýstingur vetnis) á framleiðslu lokaafurða. Einnig voru áhrif utanaðkomandi elektrónuþega könnuð á framleiðslu afurða hjá stofni AK_5 við niðurbrot á glúkósa. Hámarks etanólframleiðslugeta stofns AK_5 er 1,7 mól /mól af glúkósa; 1,35 mól etanól/mól xylósa; 7,7 mM/g sellulósa og 4,4 mM/g gras. Hægt er að sveigja stofninn nær algerlega frá etanólframleiðslu yfir í ediksýruframleiðslu með því að halda hlutþrýstingi vetnis nálægt núlli (með því að hafa utanaðkomandi elektrónuþega með í ræktun). Helsti galli stofnsins er sá að hann þolir ekki háan styrk glúkósa. Hins vegar væri hægt að komast hjá þeim galla með því að rækta hann í skammtarækt eða sírækt. Mesta vetnisframleiðslugeta stofns CMC_{15} næst við lágan hlutþrýsting vetnis og er þá 3,1 mól H_2 á hvert mól af glúkósa. Vetnisframleiðsla stofnsins á grasi, formeðhöndluðu með 0,5 % sýru og ensímum, mælist 4,83 mól H_2/g gras. Helsti galli stofnsins er sá að hann virðist þola illa lækkun á sýrustigi samfara ediksýruframleiðslu sem leiðir til þess að hann brýtur eingöngu niður hluta hvarfefnanna. Besta leiðin til að komast hjá þeim vanda væri að vera með stofninn í sírækt þar sem stöðugu kjörsýrustigi stofnsins er viðhaldið.

Lykilorð: *Thermoanaerobacter*, hitakær, etanól, vetni, flókinn lífmassi,

Table of contents

List of figures

List of tables

1 Research objectives

The objective of this research was to investigate the ability of two anaerobic, thermophilic bacteria to produce ethanol and hydrogen from various carbon substrates present in complex biomass. The strains were isolated from Grensdalur (SW-Iceland) and originate from the strain collection of the University of Akureyri, Iceland. Fifteen strains were cultivated on glucose, xylose and hydrolysates from Whatman paper (cellulose) and grass. Based on the ability of the strains to ferment these substrates and the end product formation, two strains were selected for further studies. One strain showed promising ethanol yields and the other showed promising hydrogen yields. The results are presented as two manuscripts of scientific papers (Chapters 5 and 6).

2 Introduction

The world´s energy demand is constantly increasing; the primary energy production has increased from $6.31 \cdot 10^7$ GWh/year in 1970 to $1.44 \cdot 10^8$ in 2008 or more than doubled in less than 40 years (Figure 1) and projections indicate that this trend will continue into the foreseeable future (EIA, 2009).

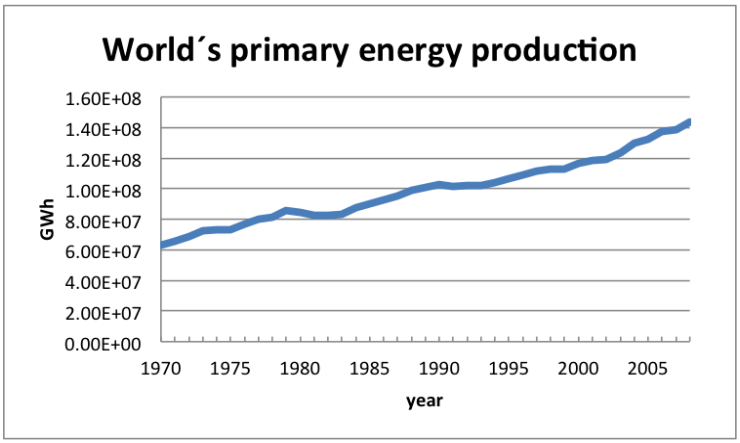

Figure 1 World primary energy production 1970 to 2008 (EIA, 2009)

Table 1 shows that over 85% of the world´s primary energy production is derived from fossil fuels (EIA).

Table 1 The world´s primary energy production by energy source (%)

Period	Oil, coal and gas	Nuclear energy	Renewable energy
1970-1974	92.7	0.8	6.5
1975-1979	91.2	2.0	6.8
1980-1984	88.6	3.5	7.9
1985-1989	86.7	5.5	7.8
1990-1994	85.7	6.3	8.0
1995-1999	85.1	6.5	8.4
2000-2004	85.4	6.6	8.1

This has raised concerns, not only because of the increase in carbon dioxide in the atmosphere from 355 ppm in 1990 to 391 ppm in 2011 (Mauna Loa Observatory, 2011) leading to global warming, but also health problems associated with pollutants such as fine particulate organic matter and heavy metals (Noyes, P. D. *et al*, 2009; Kumaresan *et al*, 2011), as well as to the finite nature of these resources.

When predicting the quantity of oil supplies, Hubbert´s Peak Theory is commonly used. The American geophysicist Marion King Hubbert based his theory on studies on how fast new oil reservoirs are discovered and on the oil production rate as well as the fact that crude oil is finite. To demonstrate this theory he presented in 1956 his well known bell shaped curve on the exhaustion of the American oil resources (Figure 2) (Hemmingsen, 2010). Hubberts predictions of the decrease in US oil production fit observations to date (EIA, 2009).

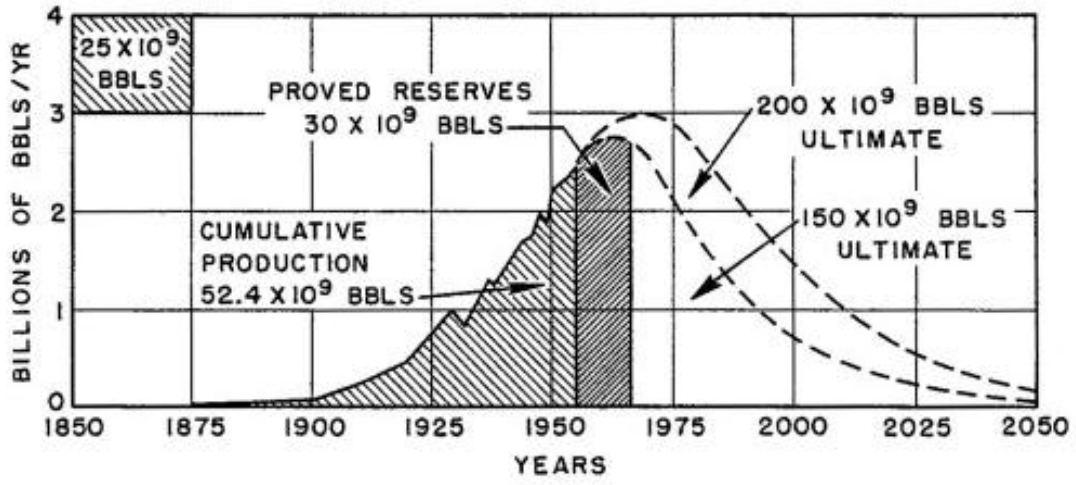

Figure 2 The exhaustion of the American oil resources (Hemmingsen, 2010)

The theory is considered to apply to smaller areas as well as to the world as a whole and it has repeatedly proved right in both smaller and larger areas (Cavallo, 2004). As some common textbooks on the subject point out, a general view is that the crude oil reservoirs might be exhausted some time in the twenty-first century (Glazer & Nikaido, 2007). Some analysts estimate that peak oil production has already been reached, whereas others estimate that peak oil consumption will occur around 2020. This is the main reason for the increased efforts in searching for renewable, carbon-free energy sources as well as renewable carbon-based alternatives (Rittmann, 2008).

2.1 Renewable energy

Renewable energy is defined as energy obtained from sources that are replenished at no less than the same rate as they are utilized (Alexander & Boyle, 2004). Today, there are several known renewable energy types although less than 15% of today´s primary energy production can be defined as renewable energy, its proportion is expected to rise significantly until the year 2100 (Panwar *et al*, 2010). The main reasons for the low proportion of renewable energy are high start up costs for its production and the low relative price of fossil fuels. Rising prices of fossil fuels together with development towards less expensive production methods and possible governmental subsidy of renewable energy start-up and production cost, will help augmentation of renewable energy ratio (Environmental Literacy Council, 2008b-c). Below, short descriptions of the main renewable energy sources are discussed.

2.1.1 Wind power

The technique of harvesting wind power to produce electricity is highly evolved and can readily compete with other electricity producing techniques. One huge advantage of using windmills for electricity production is the fact that they do not produce any CO_2 (Panwar *et al*, 2010). Wind power is used in many countries, e.g. in Denmark which produces about 20% of its electricity by using windmills.

2.1.2 Solar energy

Solar energy can be harnessed for the production of electricity and for the heating or cooling of air and water. This energy is the cleanest, most abundant, renewable energy source available. Modern technology allows us to capture this power in four ways: Photovoltaic (converting light to electricity), heating and cooling systems (solar thermal), concentrating solar power (utility scale), and lighting (Solar Energy Industries Association). The world´s solar energy electricity generation has increased more than 5 fold between 2000 and 2010 when it reached 23.899 MW (NREL, 1010).

2.1.3 Geothermal energy

Geothermal energy is energy gained from Earth´s hydrothermal systems. Water that sinks below Earths surface at low temperature geothermal areas can be used as tap water or for heating buildings. Water from high temperature geothermal areas can be used as steam to heat water or to drive turbines that produce electricity (Environmental Literacy Council, 2008c).

Geothermal areas with too little permeability for water to flow naturally through it can also be exploited for energy production. These areas are called hot dry rock and are usually exploited by leading cold water down through holes which are drilled into the ground. Steam and pressure then builds up and the steam is used to drive turbines, which in turn produce electricity. Hot dry rock geothermal areas are much more common than geothermal areas with water and steam naturally flowing through it. The total energy available at crustal hot dry rock geothermal reserves to a depth of 10 km worldwide equal to 100–1000 times the total available fossil fuel energy (Dunchane, 1994).

2.1.4 Hydroelectricity

Hydropower is produced by the motion of water and is harnessed to power electricity-producing engines. It has been utilized for a very long time, e.g. in ancient Greece, where water wheels where essential for grinding wheat into flour. The energy extracted from falling water is transformed into electricity by means of turbines and generators, led into an electricity grid and distributed to end users (US Department of Energy, 2005a-b). Due to its dependency on water constantly running, hydropower is regarded as renewable energy (Environmental Literacy Council, 2008d). The main benefits of hydropower are: high availability, no CO_2-emissions and simple technology of harnessing (US Department of Energy, 2005a; Environmental Literacy Council, 2008d). On the other hand, building hydro-electric power plants is very costly and the water lagoons occupy large areas of land which may have a negative environmental impact. Once constructed, however, the operational cost is relatively low (Environmental Literacy Council, 2008d).

2.1.5 Bioenergy

Bioenergy is energy derived from biomass, which is organic matter produced by plants and microorganisms (McNaught & Wilkinson, 2006). Various liquid and gaseous fuels can be derived from biomass (Environmental Literacy Council, 2008a) or heat. The carbon dioxide fixed by plants, algae and microorganisms can also be combusted in the presence of oxygen rendering carbon dioxide and water (Australian Institute of Energy, 2009). Direct combustion of biomass is an ancient method to gain energy but is rather inefficient due to incomplete combustion and pollution. Hence, attention has turned more recently to the conversion of biomass to less polluting, easier-to-handle fuel forms, such as hydrogen, methane and ethanol. Biomass-derived fuels are still much more expensive than fossil fuels, but emerging technologies will decrease the cost in coming years (Balat, 2010; Ni *et al*, 2006).

2.2 Biomass

Biomass is any carbon-based material produced by living or recently living organisms. Other building blocks of biomass are hydrogen, oxygen and nitrogen as well as traces of other elements such as phosphorus and sulphur (Biomass energy center). Carbohydrates, proteins and lipids are examples of biomass.

Photoautotrophic organisms, such as plants and algae, fix CO_2 from the atmosphere and convert it to sugars, starch and/or lignocellulosic biomass which in turn can be used for biofuel production. When this fuel is burnt, the CO_2 is released back to the atmosphere causing no net change in the amount of atmospheric CO_2. Burning fossil fuels, on the other hand, raises the atmospheric CO_2 ratio since that carbon dioxide originates from plant material present millions of years ago thus releasing CO_2 from long ago into the atmosphere and raising its ratio.

The composition of biomass derived from plants is highly variable depending on the type of plants. Sugar and starch-rich biomass like sugarcane and corn yield mostly simple sugars like sucrose and glucose upon hydrolysis. Lignocellulosic biomass, on the other hand, is a heteropolymer containing many different types of sugars and lignols, bound together with various chemical bonds. Therefore, extensive pretreatment methods are often necessary to convert this type of biomass to fermentable sugars (Kosaric *et al*, 2001).

2.2.1 Sugars (building blocks of biomass)

Many different types of sugars exist in nature, e.g. hexoses, pentoses disaccharides and more. Hexoses contain six carbon molecules whereas pentoses contain five carbon molecules. These sugars can form chemical bonds between them, forming di- or polysaccharides but they can also be found as monosugars in nature.

Most fermenting microorganisms can ferment glucose via the Embden-Meyerhof (EM) pathway or the Entner Doudoroff (ED) pathway (less common) (Lynd *et al*, 2002).

The sugars most frequently used for biofuel production are glucose and fructose. Sugarcane and corn are e.g. utilized for ethanol production with fermenting microorganisms as they contain abundant sucrose and glucose, respectively.

2.2.2 Starch

Starch consists of two major fractions, i.e. a water soluble amylose (Figure 3), and a water insoluble fraction, amylopectin, with a higher molecular weight (Figure 4). Amylose is a linear chain of 200 to 500 glucose units connected with α-1,4-O-glycosidic bonds but amylopectin consists of glucose units connected in a chain containing side branches connected by α-1,6-O-glycosidic bonds (Grey *et al*,, 2006; Stevenbø *et al*, 2006; McMutty &Simanek, 2007). Starch can be digested by humans.

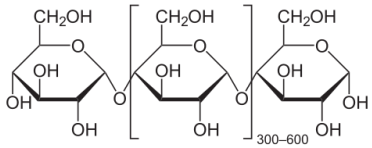

Figure 3 Amylose

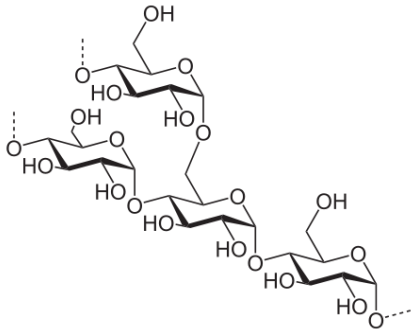

Figure 4 Amylopectin

Sweet potatoes, rice and corn are examples of starch-rich plants which can and have been used for ethanol production (Kosaric *et al*, 2001; Chang *et al*, 2010). Corn is for instance the main source for bioethanol production in the USA (Renewable Fuel Association, 2010). The fact that raw material for food and feed is used for fuel production has provoked a worldwide food vs. fuel debate.

Four enzymes are needed for a completed degradation of amylose and amylopectin to glucose. The enzymes α-amylase and β-amylase hydrolysate the α-1.4-O-glycosidic bonds of the amylose and within the chains of the amylopectin leaving a mixture of glucose, maltose and isomaltose. Maltose is a disaccharide of glucose units connected

with a α-1,4-O-glycosidic bond and isomaltose is a disaccaride of glucose units connected with a α-1,6-O-glycosidic bond. The enzyme amylo-1,6-O-glucosidase breaks the α-1,6-bonds of the isomaltose and the enzyme maltase breaks the α-1,4-glycosidic bond of the maltose (Perry, Staley & Lory, 2002).

2.2.3 Lignocellulose

Lignocellulose (Figure 5) is a complex biomass, from plants, composed of cellulose, hemicellulose and lignin, It constitutes about 50% of Earth‘s biomass. It is the largest source of renewable carbon on Earth and is therefore interesting as a substrate for biofuel production. The composition of lignocellulose varies (cellulose, 40 – 55%, hemicellulose 25 – 50% and lignin 10 – 40%) depending on the origin of the biomass, e.g. hardwood, softwood or grasses. These three different polymers bind to each other with hydrogen bonds with covalent cross-links that form the complex lignocellulose which makes up more than 90% of the dry weight of plant cells (Balat, 2010).

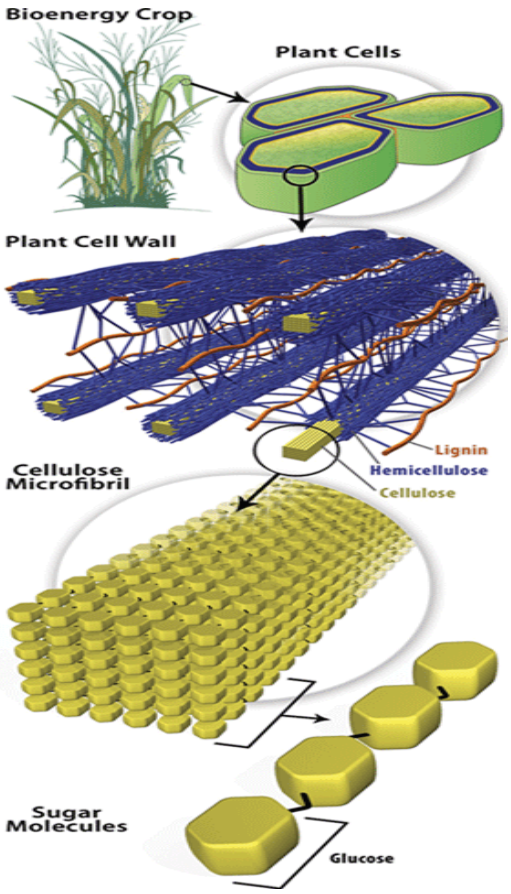

Figure 5 Lignocellulose (Bioenergy, 2008)

2.2.3.1 Cellulose

Cellulose is the most abundant organic substance on Earth. It is composed of thousands of β-1,4 linked glucose units that form long, linear, chains (Monserrate *et al*, 2001) (Figure 6). Hydrogen bonds within the cellulose chains and between adjacent chains strengthen the cellulose. It has both highly organized crystalline regions and less organized amorphous regions. Cellulose has a high tensile strength; it cannot be dissolved in water and is highly resistant to degradation compared to starch. Although glucose is also the only sugar present in cellulose, it cannot be digested by humans (Kosaric *et al*, 2001) because of the β-1,4 linkages instead of α-1,4-glycosidic linkage in starch.

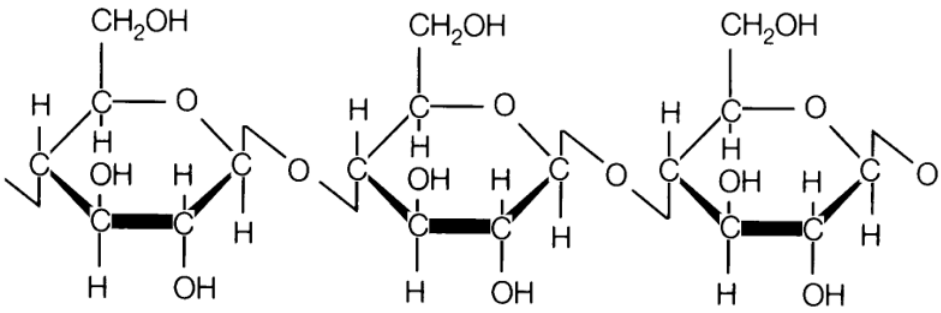

Figure 6 β-1-4 linked glucose units of cellulose

2.2.3.2 Hemicellulose

Hemicellulose is a heteropolysaccharide with a structural homology to cellulose but the backbone is made of 1,4-linked β-D-pyranosyl units. It consists of many types of sugars, e.g. pentoses (D-xylose and L-arabinose), hexoses (L-galactose, D-mannose, L-rhamnose, L-fructose) and D-glucuronic acid as well as acetyl groups (Figure 7). The sugars of hemicellulose form complex, branched chains but show a much lower degree of polymerization as compared to cellulose (Glazer & Nikaido, 2007). The types of sugars and the ratio between them in the hemicellulose differ between plants. Hemicellulose is classified into three main groups, depending on the most common sugar present, namely: xylan, mannan and galactan (Bringham *et al*, 1996).

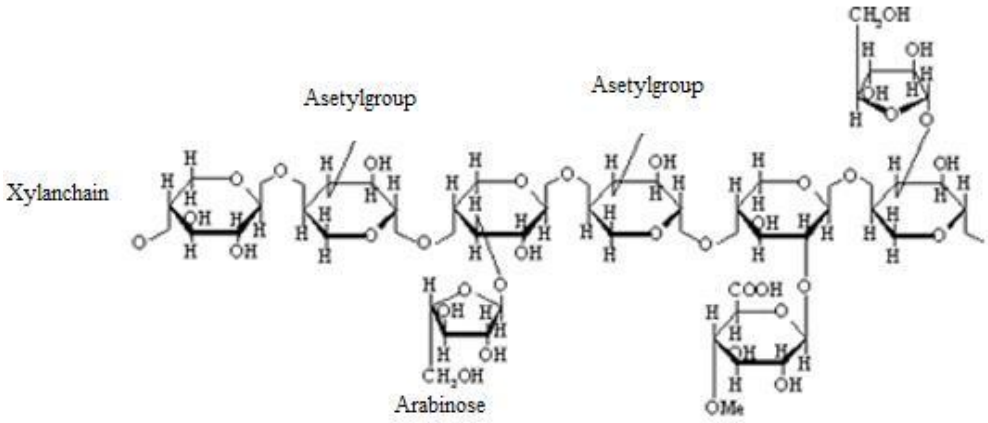

Figure 7 Example of a hemicellulose structure

2.2.3.3 Lignin

Unlike cellulose and hemicellulose, lignin is a random heteropolymer bound together with different types of chemical bonds. It is the most abundant aromatic compound on Earth. It is an amorphous copolymer made of phenylpropane derivatives (Figure 8).

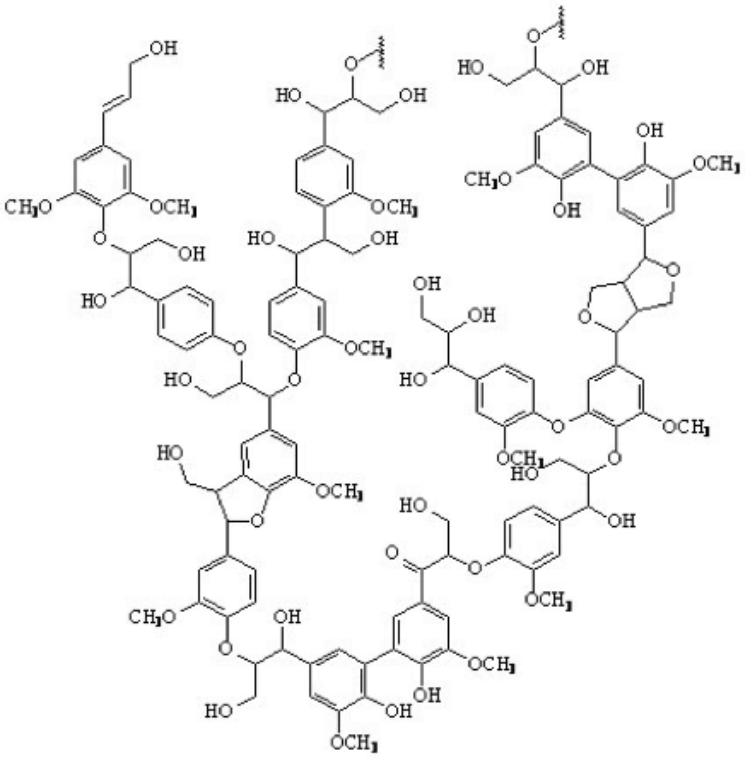

Figure 8 An example of lignin structure

The precursors of lignin are three alcohols derived from *p*-hydroxycinnamic acid; *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Galletti & Bocchini, 1995; Hendriks & Zeeman, 2008; Salo *et al*, 1989) (Figure 9). Lignin is divided into three categories: softwood lignin, hardwood lignin and grass lignin. Softwood lignin is predominantly polymers of coniferyl alcohol. Hardwood lignin is a mixture of coniferyl, and sinapyl alcohol in equal amounts. Grass lignin contains all of the three main chemicals mentioned above (Glazer & Nikaido, 2007).

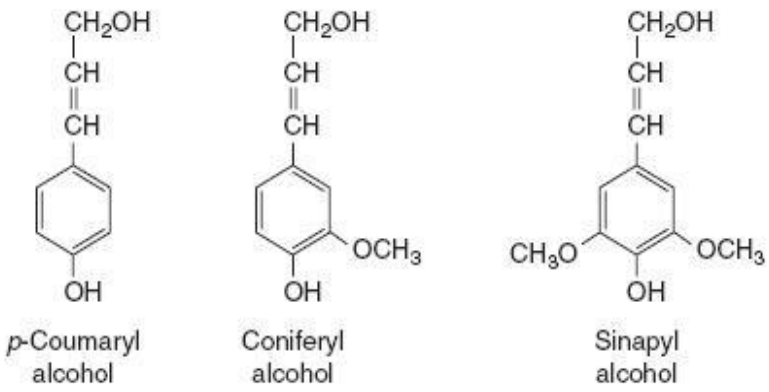

Figure 9 The three main precursors of lignin

Lignin lends rigidity and great strength to plants. An active plant contains hollow spaces, between the cellulose– and hemicellulose chains in the cell wall, for transport. As the plant grows older these hollow spaces fill up with lignin (e. lignification), thus making the plant rigid. This process enables some trees to grow up to, or even above, 100 meters in height (Glazer & Nikaido, 2007). Lignin formation is also responsible for the impermeability of plants and protection against microbial attacks and invasion of oxygen and it is the biggest source of aromatic compounds on Earth (Hendriks & Zeeman, 2008).

Only few organisms in nature are known for degrading lignin, e.g. white-rot fungi. None of them utilize lignin for energy but merely use it as a carbon source (Fackler *et al*, 2006; Perry *et al*, 2002).

2.3 Fermentation

Organisms have three main ways of gaining energy: through photosynthesis, fermentation and respiration. Fermentation takes place at anaerobic conditions, whereas respiration relies on the presence of oxygen. The energy harvested from these processes is stored as ATP (adenosine triphosphate). In fermentation ATP is a product of a series of oxidation and reduction reactions, where energy is released from organic compounds. Respiration is superior to fermentation regarding energy production, yielding up to 38 ATPs from glucose oxidation compared to only 2 ATPs from fermentation (Lehninger *et al*, 2005).

Glycolysis is the first step of breaking down glucose, for both aerobic and anaerobic organisms (Figure 10). It consists of two main phases: preparatory reactions, which require 2 ATPs, and oxidation reactions, where 4 ATPs are formed yielding a net total of 2 ATPs and two molecules of pyruvate. Additionally, two molecules of NADH are produced from one mole of glucose through glycolysis.

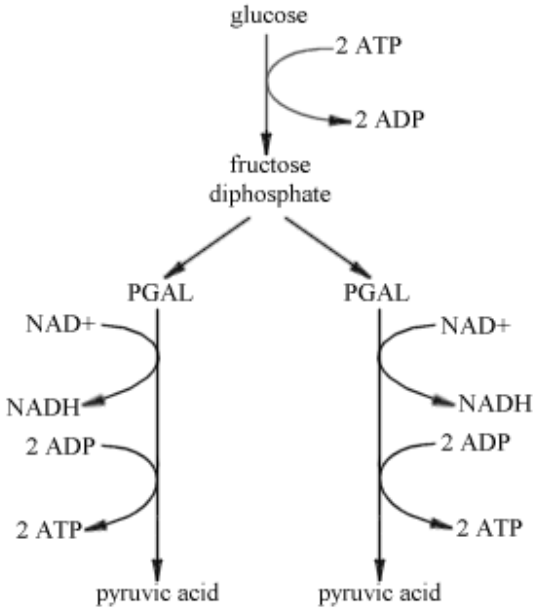

Figure 10 Glycolysis

A less common pathway for glucose degradation is the Entner Doudoroff pathway (ED). The net production of ATPs by ED is only one ATP. An example of a bacterium that uses ED is the powerful ethanol producer *Zymomonas mobilis*.

After glycolysis, the most profitable way for an organism is to degrade pyruvate completely to carbon dioxide by the citric acid cycle and channel the electrons through the respiratory chain. However, this is only possible when oxygen is present as the terminal electron acceptor, since under anaerobic conditions NADH needs to be reoxidized back to NAD^+ by reducing pyruvate to reduced end products (e.g. ethanol, lactate, hydrogen, butyrate) without ATP formation (Figure 11).

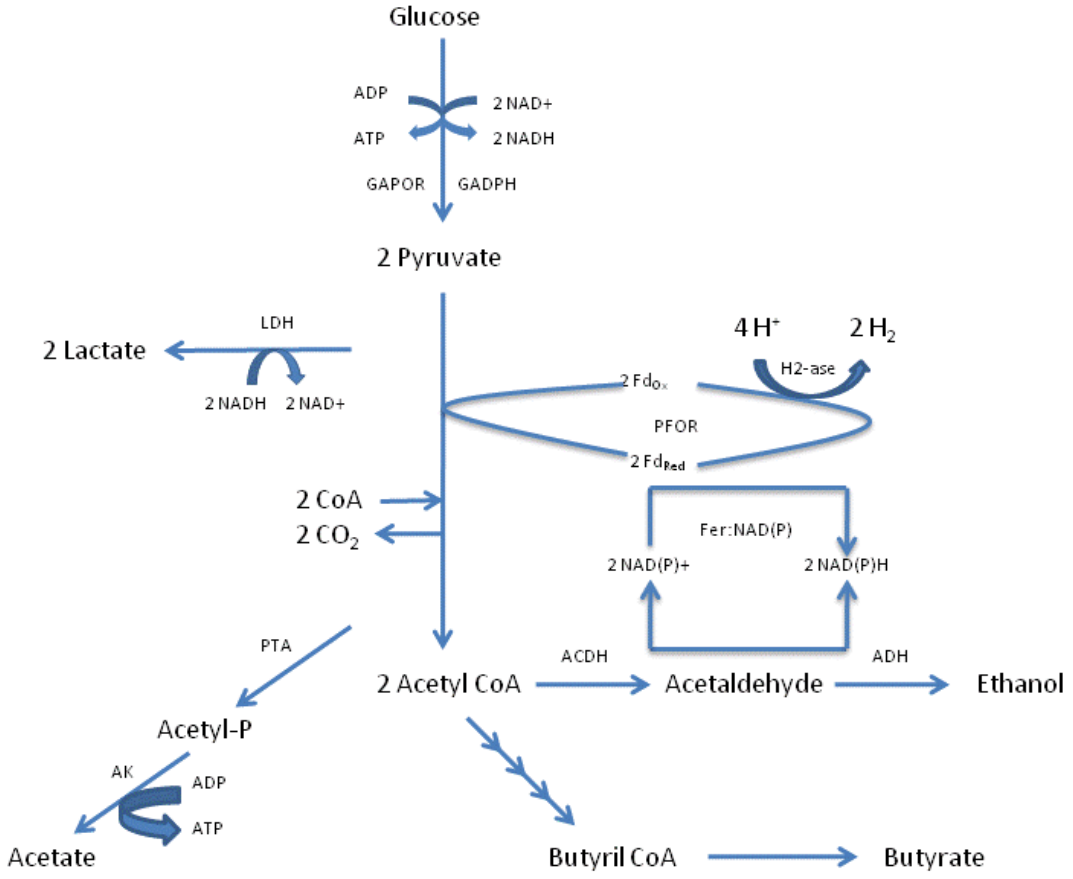

Figure 11 Glucose degradation to various end products by strict anaerobic bacteria (Sveinsdottir et al., 2011)

2.4 Pretreatment of biomass

As mentioned earlier, lignocellulosic biomass consists of many different types of sugars and aromatic compounds which are tightly bound together with various types of chemical bonds. Many microorganisms are equipped with various enzymes to break down these components. Some thermophilic bacteria, such as *Clostridium thermocellum, Caldocellum saccharolyticum* and *Acidothermus cellulolyticus* are known to have cellulolytic and/or hemicellulolytic enzymes for biomass hydrolysis (Chang & Yao, 2011; Bergquist *et al*, 1999; Gibbs *et al*, 1992; Te'o *et al*, 1995). However, the yields from microbial degradation of untreated complex biomass are usually much lower as compared to the yields obtained after various pretreatments. Therefore, biomass is usually pretreated to release the sugars (Figure 12), which in turn are fermented by fermenting microorganisms, to various biofuels. The aim of the pretreatment is to separate the lignin from the rest of the biomass and secondly to break the bonds within the cellulose and the hemicellulose to free the sugars and make them more accessible to the fermenting organisms. These pretreatments can be physical, chemical, biological or a mixture of those (Stephanopoulos, 2007).

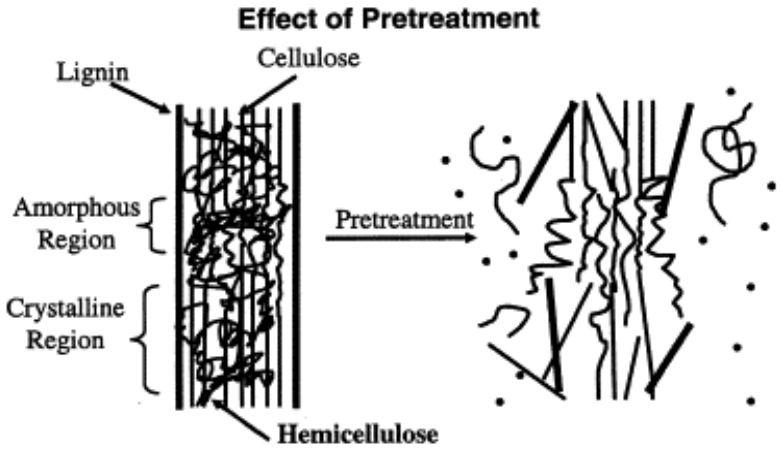

Figure 12 Effect of pretreatment on lignocellulosic material (Hsu et al., 1980)

Myriad pretreatment methods have been developed and tested under various conditions. Some of the most common ones are described in the following subchapters.

2.4.1 Physical pretreatment

The objective of physical pretreatments is to decrease the particle size of the biomass treated and increase its surface to volume ratio. This can be done by dry and/or wet cutting and grinding (da Costa Sousa *et al*, 2009; Tassinari *et al*. 1980).

2.4.2 Physio-chemical pretreatment

Steam explosion is a physio-chemical pretreatment that works well on biomass (particularly hardwoods). Hacked, wet biomass is heated up to 160 – 260°C under a pressure of 0.69-4.83 Mpa. After few minutes, the pressure is abruptly released causing the steam to explode the biomass from within (Brownell & Saddler, 1984; Sun *et al*, 2002). This method does not use any chemicals other than water and presents therefore no environmental costs. It is considered to be the most cost effective pretreatment for hardwoods and agricultural residues but is less effective on softwoods (Clark *et al*, 1987).

Less common physio-chemical pretreatments include ammonia fiber explosion and CO_2 explosion. Those methods use the same principles as steam explosion; they use liquid ammonia and CO_2, respectively, which requiers recycling of the pretreatment agents to minimize the environmental impact and lower costs (Zeng *et al*, 1998).

Liquid hot water pretreatment is performed by heating the biomass in water up to 200 – 230°C under enough pressure to keep the water in liquid state. After about 15 minutes in the hot water, 40 – 60% of the biomass is dissolved depending on the type of biomass (Mok & Antal, 1992 & 1994; Mosier, *et al*, 2005).

2.4.3 Chemical pretreatment

Many different chemical pretreatments have been developed. The most common are acidic and alkaline pretreatments covered in sections 2.2.3.1 and 2.2.3.2, respectively (da Costa Sousa *et al*, 2009).

2.4.3.1 Acidic pretreatments

Acid causes chemical hydrolysis and is most effective on hemicellulose and lignin giving the enzymes a better access to the cellulose in the next steps (Weil *et al*, 2002). Both concentrated and dilute acids can be used. The use of concentrated acids can be problematic since they are very toxic and corrosive, requiring corrosion resistant reactors (Sivers, *et al*, 1995). A more favorable and thus more common method is using diluted acids, usually hydrochloric acid (HCl) (Esteghlalian, *et al*, 1997). Two different implementations have been reported for dilute acid pretreatment; high temperature (T > 160°C), continuous-flow process for low solids loading (Brennan, *et al*, 1986; Converse *et al*, 1989); and in low temperature (T < 160°C), batch process for high solids loading (Cahela *et al*, 1983; Esteghalian *et al*, 1997).

2.4.3.2 Alkaline pretreatments

Alkaline pretreatment is mainly used to solubilize and extract lignin from the biomass. It is based on alkaline catalysts, such as ammonia or sodium hydroxide that attack and cleave acetyl groups and hydrolyze lignin–carbohydrate ester linkages (Chang & Holtzapple, 2000). The wet biomass plus the alkaline catalyst is heated under pressure. When ammonia is used as the catalyst, no organic acids (which are major inhibitors in fermentation) form (Lau *et al*, 2008).

2.4.4 Biological pretreatments

Biological pretreatment can be performed using fungi that release extracellular enzymes that can remove lignin from the biomass (Fan *et al*, 1987). A disadvantage to using this method is that it is relatively time consuming (hours to days) and that the fungi may consume part of the sugars released (Christian *et al*, 2005). The addition of

the enzymes alone to the wet biomass allows them to operate at their optimal temperature and pH. Enzymes are usually highly specific and many cellulolytic and polymer degrading enzymes produced by aerobic and anaerobic bacteria and fungi have been identified. The enzymes most commonly used in the industrial enzymatic pretreatment of biomass are produced by the fungi *Trichoderma reesei*. Particularly endoglucanase, which randomly attacks the β-1,4-glucan linkages of cellulose, exoglucanase, which releases cellobiose from the non-reducing ends of the cellulose chain and cellobiase which hydrolyzes cellobiose to glucose (Glazer & Nikado, 2007; Lee, 1997). Examples of enzymes able to degrade polymers of hemicelluloses are glucuronidase, acetylesterase, xylanase, β-xylosidase, galactomannanase and glucomannanase (Duff & Murray, 1996). The main benefits of using enzymatic pretreatments are the mild environmental conditions used minimizing the corrosion problems that are often associated with using acid or alkaline pretreatments, their use in catalytic amounts, and their renewable nature (Faber, 2004; Bornscheuer & Kazlauskas, 2006). The main drawback of using enzymes is their high cost (Duff & Murray, 1996).

2.4.5 Inhibitory compounds formed during pretreatments

During pretreatment, compounds that can inhibit enzymatic hydrolysis as well as fermentation, can be released and/or produced. The main inhibitory compounds that are byproducts of biomass pretreatments are furfural, 5-hydroxymethylfurfural, phenolic acids and aldehydes, levulinic acid, and other aliphatic acids (Weil *et al*, 2002).

2.5 Ethanol production

The first records of ethanol (EtOH) being used as a fuel are from 1908 when a car produced by Henry Ford ran on a mixture of gasoline and ethanol (Gottemoeller & Gottemoeller, 2007). Ethanol as vehicle fuel was, however, not produced from biomass until the early 1930´s, when it was made from sugarcane in Brazil. EtOH production reached its peak at 77 million liters in Brazil during World War II (Nardon & Aten, 2008). Interest in EtOH declined after the war, as it was outcompeted by cheap oil, but rose again during the oil crisis in the 1970´s. EtOH production from starch based plant materials has been rising steadily in the US since the late 1970´s (Nass *et al*, 2007).

The worlds mineral oil reservoirs are slowly being depleted and oil has to be extracted from more remote areas at higher costs. This, along with steadily increasing demands leads to rising oil prices which makes alternative fuel sources, like ethanol more attractive. Since 1975, there has been a great emphasis on ethanol production from sugarcane in Brazil, and since the late 1970´s there has been a steady increase in ethanol production from starch-based plant material in the US (Nass *et al*, 2007).

In the year 2010, the US and Brazil produced more than 65.3 billion liters of EtOH which accounts for 89% of the world´s production (Renewable Fuel Association, 2010).

The majority of ethanol production today is achieved from the fermentation of the disaccharide sucrose (sugarcane) and the monosaccharide glucose (starch). The yeast *Saccharomyces cerevisae* is primarily used to ferment these sugars to ethanol. The properties of *S. cerevisiae* that make it suitable for this task are: high ethanol yields (>1.9 mol ethanol/mol hexose), high osmotolerance (tolerates > 12% ethanol) and resistance to many toxic inhibitors (Jeffries, 2006). The biggest drawback of *S. cerevisae* is that it has a very narrow substrate range. Its wild type can only degrade glucose, fructose and sucrose.

Another highly efficient ethanol producing microorganism is the anaerobic, mesophilic bacterium, *Zymomonas mobilis*. It was isolated from agave sap in Mexico, palm saps in Africa and Asia, and sugarcane in Brazil after it attracted microbiologists

attention in 1912 because of fermented apple juice (Glazer & Nikaido, 2007). Historically, it has been used to make alcoholic beverages from plant sap in tropical areas (Yanase *et al*, 2007). *Z. mobilis* has a limited substrate spectrum; it degrades sucrose, glucose and fructose, has a high osmotolerance and can withstand up to 13% ethanol by volume. The bacterium can grow in the presence of up to 40% glucose (≈ 2.5 M), which is not surprising since it is isolated from such high sugar environments. *Z. mobilis* produces ethanol via the Entner-Doudoroff (ED) pathway; its ethanol efficiency on glucose is up to 97% and it grows 2.5 - 4 times faster than *S. cerevisae* (Bai *et al*, 2008; Rogers *et al*, 1982). The biggest shortcoming of *Z. mobilis* is its narrow substrate range, as the wild type *Z. mobilis* can only degrade glucose, fructose and sucrose (Swings and De Ley, 1977). Also,the fact that the enzymes responsible for breaking down sucrose can also polymerize it, causes a significant reduction in ethanol yields (Liu et al., 2010). Genes responsible for xylose and arabinose degradation have been introduced into *Z. mobilis* allowing the bacterium to convert xylose to ethanol (Joachimsthal and Rogers, 2000; Lawford & Rousseau, 2002).

Ethanol produced from simple sugars is called 1st-generation production because this type of substrate can also be used for food and feed. This has been highly criticized and has led to a worldwide “food versus fuel” debate which has led to an increasing interest in other types of biomass for ethanol production (Srinivasan, 2008). However, complex lignocellulosic biomass cannot be used for human consumption and it is therefore an interesting alternative substrate for ethanol production. This has led to increased research within this field for the last 10 to 15 years. Ethanol production from this type of biomass is referred to as 2nd-generation ethanol production.

As mentioned earlier, lignucellulosic biomass consists of many different types of hexoses, pentoses and aromatic compounds bound together with different types of chemical bonds. The wild type of the *S. cerevisae* can only degrade glucose, fructose and sucrose but no pentoses which are present in large amounts in lignocellulosic biomass. Therefore, it is unsuitable for degradation of lignocellulosic biomass (Jeffries, 2006). Genetic engineering has been used to introduce genes, coding for enzymes that degrade arabinose and xylose (van Maris *et al*., 2007) but it has been difficult to obtain stable cultures (Sanchez *et al*, 2010; Wisselink *et al*., 2008).

A search for a suitable microorganism for ethanol production from complex biomass is a worthy assignment. It has been known for long that thermophilic bacteria can ferment various carbohydrates to ethanol, among various other end-products. However, it was not until 1980 that the focus was directed to ethanol production as a fuel, using these bacteria (Ben Bassat *et al*, 1981; Lamed & Zeikus, 1980).

In general, a good ethanol producer should have the following qualities (Dien *et a*l, 2003; Glazer & Nikaido, 2007).

- Be able to hydrolyze complex biomass
- Be able to ferment many different types of sugars to ethanol
- Tolerate high concentrations of substrates
- Withstand high concentrations of ethanol
- Grow in the presence of inhibitory compounds from complex biomass
- Grow at simple and inexpensive conditions
- Grow at high temperatures and extreme pH to prevent contamination by other microorganisms

To date, no microorganism with all the above listed qualities has been found. The biggest problem with thermophilic bacteria, regarding ethanol production, is their low tolerance towards ethanol and lower ethanol yields because of the production of other end products. The yeast *S. cerevisae* and the bacterium *Z. mobilis* can grow in the presence of up to 12 and 13% (w/vol) of ethanol respectively, but most wild type thermophilic bacteria tolerate less than 3% (w/v). A number of studies on the ethanol tolerance of thermophilic bacteria have been performed as well as attempts to enhance their ethanol tolerance. A mutant strain of *Thermoanaerobacter ethanolicus* was able to tolerate up to 10% ethanol (wt/vol) and the genetically engineered highly efficient xylose degrader *Thermoanerobacter mathranii* BG1L1 was able to tolerate up to 8% (Carriera *et al*, 1983; Georgieva *et al*, 2008b). A *Clostridium thermocellum* mutant strain S7 has been reported to be able to grow in the presence of 6% ethanol (w/v) (Wang *et al*., 1983) and *Thermoanaerobacter thermohydrosulfuricus* strain 39E that was repeatedly transferred to media with increasing ethanol concentrations

(evolutionary adaptation) resulted in a mutant able to produce ethanol at up to 8.0% (wt/vol) whereas the parent strain was completely inhibited at ethanol concentrations of 2.0% (Lovitt *et al*., 1984).

2.5.1 Ethanol production from complex biomass

It has long been known that thermophilic bacteria can ferment various carbohydrates to ethanol. As the "food versus fuel" debate has become more blatant in the last couple of decades, thermophilic bacteria have gained interest as potential ethanol producers from complex biomass. The earliest investigations of thermophilic bacteria using complex biomass for ethanol production focused on *Thermoanaerobacter brockii*, *Thermoanaerobacter thermohydrosulfuricus*, *Thermoanaerobacter ethanolicus* and *Clostridium thermocellum* (Ben Bassat *et al*., 1981; Lamed & Zeikus, 1980a; 1980b; Wiegel *et al*, 1983; Lovitt *et al*, 1984). More recent investigations on ethanol production capacity from various cellulosic and lignocellulosic biomass of other *Thermoanaerobacter* species, *Clostridium* species and *Thermoanaerobacterium* have been carried out.

Ethanol production by *Clostridium thermocellum* has been tested on various cellulose and lignocellulosic biomass. The highest ethanol yields from cellulosic biomass are 8.2 mM g^{-1} from filter paper. Its yields from avicel was up to 7.2 mM g^{-1} and it showed similar yields from paddy straw, sorghum stover and corn stubs, pretreated with alkali (Balusu *et al*, 2004; 2005). None of the above mentioned investigations used higher concentrations of cellulose than 8.0 g L^{-1}. Later studies showed dramatic decrease in yields with increased substrate concentrations (Lin *et al*, 2010).

Thermoanaerobacterium sp. AK_{17}, isolated from an Icelandic hot spring was able to produce 4.0 and 8.6 mM g^{-1} on acid/alkali pretreated grass and cellulose respectically (Almarsdottir *et al*, 2012). Those yields were obtained by a substrate loading of 2.5 g L^{-1}. The yields dropped to 2.0 mM g^{-1} on grass and 5.8 mM g^{-1} on cellulose when raising the substrate loading to 7.5 g L^{-1} (Almarsdottir *et al*, 2012; Sveinsdottir *et al*, 2009).

The highest ethanol yields from complex biomass so far are 9.2 mM g^{-1} from corn stover and wheat straw pretreated with acid or wet oxidation. This was achieved by

the strain *Thermoanaerobacterium* BG1L1 (Georgieva & Ahring, 2007; Georgieva *et al*, 2008a).

2.6 Hydrogen production

The hydrogen molecule carries a high energy content (≈140 MJ/kg) and its combustion results in water as the only product. This, as well as the fact that hydrogen is the most abundant element on Earth, makes it very interesting as an energy carrier and has ignited research of this biofuel.

Most of the hydrogen produced today is derived from natural gases (hydrocarbons) via steam methane reforming (Turner, 2004). The steam reformation of methane is divided into two steps. The first step (Eq. 1), where the hydrogen is formed, occurs typically at temperatures 800°- 1000°C and 20 – 35 atm but the later one (Eq. 2), where CO is converted to CO2, occurs at 200 – 400°C (Fernández, *et al*, 2012).

$$CH_4(g) + H_2O(g) \leftrightarrows CO(g) + 3H_2(g),\ \Delta H298K = +206\ kJ/mol \quad (1)$$

$$CO(g) + H_2O(g) \leftrightarrows CO2(g) + H_2(g),\ \Delta H298K = -41\ kJ/mol \quad (2)$$

Hydrogen can also be produced by electrolysis of water (Zeng & Zang, 2009) and biologically. As with ethanol, first generation hydrogen production refers to its production from sugars and sugar based biomass whereas second generation production is from lignocellulosic biomass.

2.6.1 Electrolysis

Hydrogen can be produced by leading an electrical current through water. This breaks the chemical bonds between the oxygen and the hydrogen of the water molecules yielding hydrogen and oxygen. Hydrogen is drawn towards the positively charged cathode while the oxygen moves towards the negatively charged anode (Leroy, 1983). Figure 13 shows a basic water electrolysis system.

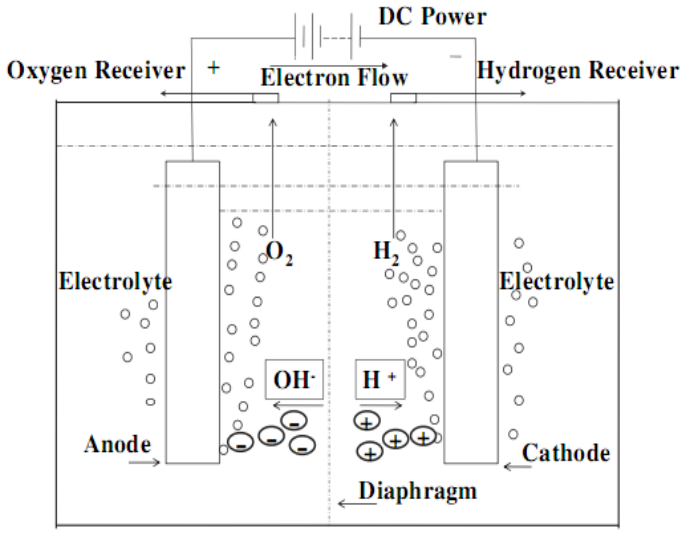

Figure 13 A basic water electrolysis system (Zeng & Zhang, 2009)

Water is indeed a renewable resource and there is plenty of it. The downside of this method is, however, that only a fraction of the energy put into the electrolysis is recovered in the hydrogen produced.

2.6.2 Hydrogen production by microbes

Microbes can produce hydrogen in four different ways: direct photolysis, indirect photolysis, photofermentation and fermentation. Hydrogen produced by microbes is called biohydrogen (Kotay *et al*, 2008).

2.6.2.1 Direct photolysis

Some green algae and cyanobacteria that absorb photons from the sunlight, use the energy from them to split water and generate reduced ferredoxin to drive the reduction of protons to hydrogen. Two photosynthetic systems (PSII and PSI) are needed for photosynthesis. The electrons that are generated when PSII adsorbs light energy, are transferred to ferredoxin (Fd) using the solar energy absorbed by PSI. Green algae and cyanobacteria possess a hydrogenase that accepts the electrons from the ferredoxin to produce hydrogen (Figure 14). Green plants do not possess the

hydrogenase and can therefore not produce H_2, instead they reduce CO_2 (Ni *et al*., 2006). A downside to this method is that it requires a large surface area. (Hallenbeck *et al*, 1978; Miyamoto *et al*, 1979).

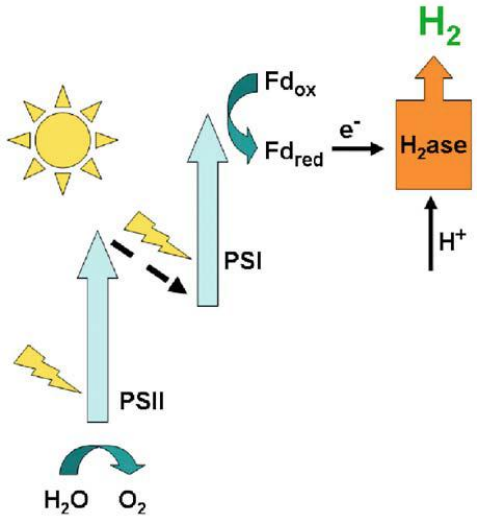

Figure 14 Biophotolysis (Hallenbeck et al., 2009)

2.6.2.2 Indirect photolysis

Some microorganisms can produce hydrogen by indirect photolysis (Benneman, 2000). This production consists of two main stages separated in space and time (Figure 15). The first step uses the same two photosynthetic systems (PSII and PSI) to generate electrons and transfer them to ferredoxin, but instead of producing hydrogen via hydrogenase, carbohydrates are produced and stored. Later, in the second step, these stored carbohydrates are converted to hydrogen and carbon dioxide in a light driven process under oxygen-depleted conditions.

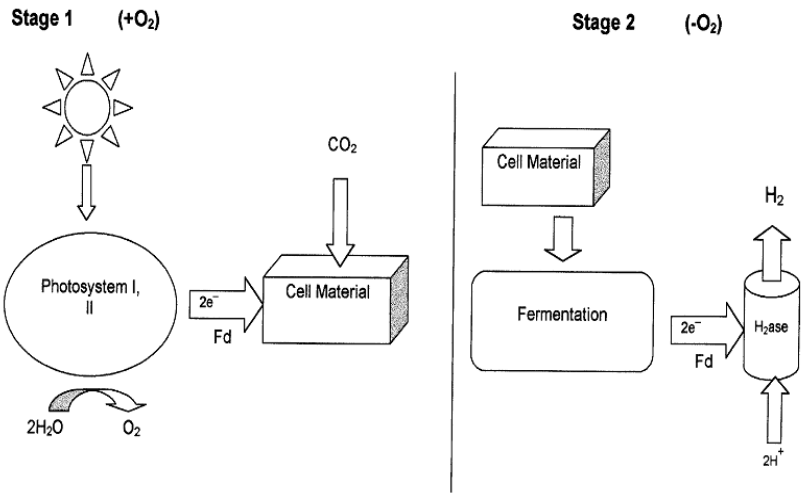

Figure 15 Indirect photolysis

Cyanobacteria, such as *Aphanocapsa montana, Anabaena variabilis* and *Spirulina* have been reported to produce hydrogen via indirect photolysis (Benneman, 2000).

2.6.2.3 Photofermentation

Photofermenting bacteria can produce hydrogen from various substrates, especially organic acids, using the energy from the light of the sun (Miyamoto *et al*, 1979; Yetis *et al*, 2000) (Figure 16).

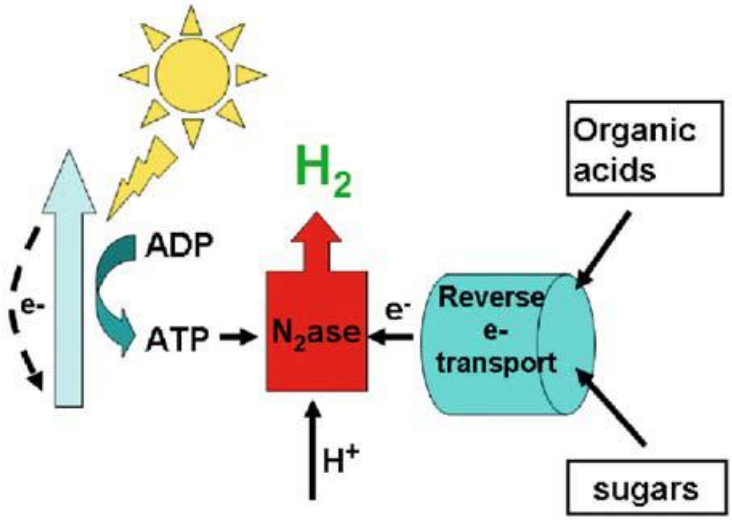

Figure 16 Photofermentation (Hallenbeck et al, 2009)

The main disadvantage of this method of producing hydrogen is that complex biomass usually needs to be pretreated before fed to the bacteria, high concentration of biomass diminishes light diffusion into the bioreactor and the reactor surface area needs to be large (Koku *et al*, 2003).

2.6.2.4 Fermentation

It has long been known that hydrogen can be produced by growing anaerobic bacteria on carbohydrate-rich substrates in the absence of light (dark fermentation). As mentioned earlier, fermentation of such substrates leads to the production of various end products (ethanol, lactate, butyrate, hydrogen and carbon dioxide) depending on environmental conditions and the microbes involved. Early investigations were mostly on mesophilic bacteria within the genera of *Enterobacter*, *Citrobacter* (Liu *et al*, 2003; Nakashimata *et al*, 2002; Oh *et al*, 2003) and *Clostridium*. The production of hydrogen is however thermodynamically more favorable at higher temperatures and the variety in end-products is less. Recently, the production of H_2 by thermophiles has received attention (van Groenestijn *et al*, 2002; van Niel *et al*, 2003).

Figure 17 shows the main degradation pathways of fermenting microorganisms resulting in different end products.

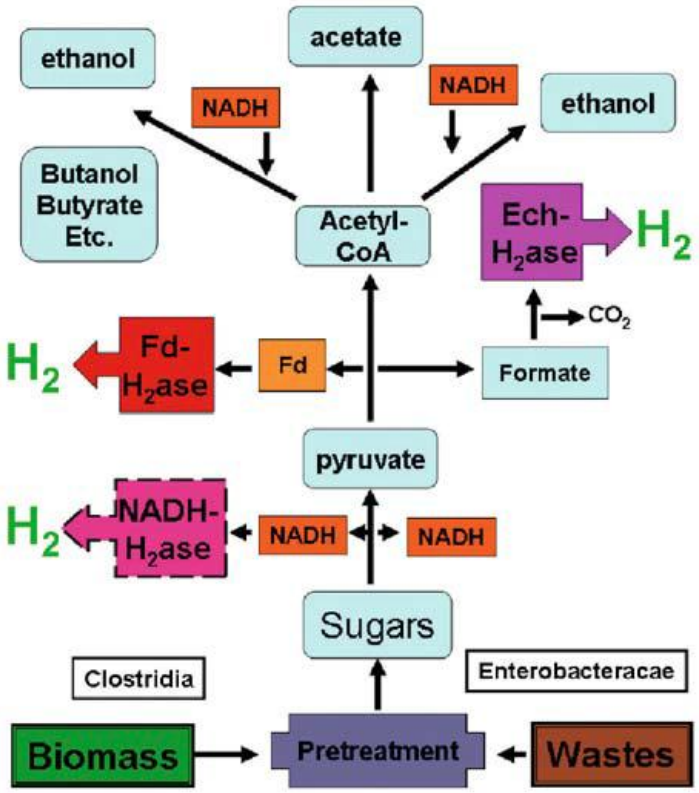

Figure 17 Dark fermentation (Hallenbeck et al., 2009)

The amount of H_2 produced by a fermenting microorganism depends on the fermentation pathways it uses. If it produces acetate, the theoretical yield of hydrogen is 4 moles per mole glucose according to the following equation (Argun *et al*, 2008).

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

If the final product is butyric acid, the theoretical yield of H_2 is two moles of H_2 per mole of glucose (Levin *et al*, 2004):

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

No hydrogen is produced if the end products are ethanol or lactate (Koskinen *et al*, 2008):

$$C_6H_{12}O_6 + 2\ H_2O \rightarrow 2CH_3CH_2OH + 2HCO_3^- + 2H^+ \quad (3)$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH + 2H^+ \quad (4)$$

In recent years, because of thermodynamic interactions, thermophilic and extremophilic bacteria (*Thermoanaerobacterium, Caldicellulosiruptor* and *Thermotoga)* have been most extensively studied for hydrogen production. Batch experiments on the H_2 production capacity of *Thermotoga neopolitana* from glucose and xylose have shown yields of 3.85 and 2.20 mol H_2 mol sugar^{-1} respectively. The experiments were carried out with sugar concentrations of 5 to 7 g L^{-1} (Eriksen *et al*, 2008; d'Ippolito *et al*, 2010; Nguyen *et al*, 2008, 2010a,b,c; Munro *et al*, 2009). *Thermotoga elfii* and *Thermotoga maritima* showed yields from 1.67 to 4.00 mol H_2 mol glucose^{-1} (Nguyen *et al*, 2008; Schröder *et al*, 1994; van Niel *et al*, 2002).

Caldicellulosiruptor saccharolyticus produced 6.6 mol H_2 mol sucrose^{-1} (= 3.3 mol H_2 mol hexose^{-1}) (van Niel *et al*., 2002) and 3.6 mol H_2 mol glucose^{-1} (Vrije *et al*, 2007). *Caldicellulosiruptor owensis* has been reported to produce 3.8 and 2.7 mol H_2 mol sugar^{-1} from glucose and xylose respectively in continuous cultures (Zeidan & van Niel, 2010).

A batch culture of *Thermoanaerobacter tengcongensis*, continuously flushed with N_2 in order to keep the partial pressure of H_2 low, resulted in almost all the glucose converted to acetate indicating the formation of 4.0 mol H_2 per mol glucose degraded (Soboh *et al*, 2004).

Among other thermophilic bacteria that have shown interesting hydrogen yields are *Clostridium* sp. (Almarsdottir *et al*, 2010 ; Levin *et al*, 2006), *Thermoanerobacterium saccharolyticum* (Cao *et al*, 2010; Kadar *et al*, 2004) and *Pyrococcus furiosus* (Schicho *et al*, 1993).

2.6.2.5 Hydrogen production from complex biomass

Studies on hydrogen production from complex biomass has proliferated recently and available data on H_2 production from complex biomass has exploded in the last three years. Various types of complex biomass have been used for thermophilic biohydrogen production in both laboratory and pilot scale processes. Table 2 shows selected literature on highest yields of hydrogen production in batch cultivation from complex biomass. The most dominant bacteria studied belong to the extremophiles often with hydrogen yields up to 4 moles of hydrogen from glucose equivalents.

Table 2 H_2 production in batch cultures from agricultural wastes and energy crops

Organisms	Feedstock	H_2 yield (mol H_2 mol glc^{-1} equiv.)	Temp (C°)	Reference
Caldicellulosiruptor saccharolyticus	paper sludge	3.70	70	Kadar *et al*, 2004
Caldicellulosiruptor thermocellum	Delignified wood fibers	1.00-2.30	60	Levin *et al*, 2006
Caldicellulosiruptor thermocellum 27405	Cellulose	0.08-2.00	60	Levin *et al*, 2006
Thermotoga neapolitana	Microcrystalline cellulose	1.00-2.20	80	Nguyen *et al*, 2008b
Thermoanaerobacterium thermosaccharolyticum	Miscanthus hydrolysate	3.40	72	Vrije *et al*, 2009
Thermotoga neapolitana	Miscanthus hydrolysate	3.20	80	Vrije *et al*, 2009
Caldicellulosiruptor saccharolyticus	Wheat straw	3.8	70	Ivanova *et al*, 2009
Caldicellulosiruptor saccharolyticus	Maize leaves	3.67	70	Ivanova *et al*, 2009

2.7 Ethanol and hydrogen producing thermophilic bacteria

Thermophilic bacteria have gained increased interest in the last two decades because of their ability to produce hydrogen and ethanol from biomass. Thermophiles can be found in many places in nature where temperatures are relatively high. Thermophilic bacteria are divided into moderate thermophiles 55 – 75°C opt, true thermophiles 55 – 75°C opt and extremophiles > 75°C (Brock, 1986). Hot springs are an example of a thermophile habitat that provide an environment with a stable temperature (Brock, 1986; Kristjansson & Alfredsson, 1986). High temperature fluids tend to contain little to no oxygen due to its low solubility in water. Thermophilic bacteria therefore live in surroundings with little or even no oxygen and thus are obligate or facultative anaerobes (Amend & Shock, 2001), with chemolitho- or heterotrophic metabolism. Fermentaion of organic material by these microbes results in the formation of various end products, e.g. acetate, ethanol, butyrate, lactate and hydrogen (Madigan *et al*, 2003).

The bacteria known as the best ethanol producers today belong to the genera of *Clostridium, Thermoanaerobacter* and *Thermoanaerobacterium* and the bacteria known as the best hydrogen producers belong to the genera *Caldicellulosiruptor* and *Thermotoga* (Sveinsdottir, *et al*, 2009). Below is a short description of these genera.

2.7.1 *Clostridium*

The genus *Clostridium* belongs to the family Clostridiaceae, order Clostridiales, class Clostridia and phylum Firmicutes (LPSN, List of Prokaryotic names with Standing in Nomenclature, J.P. Euzéby). The genus *Clostridium* is a very large genus and contains over 200 validly described species. Phylogenetic analysis of *Clostridium* bacteria have shown big distances in relations within the genus and in fact it is high time that these bacteria be reclassified. About half of the species could be classified to other genera (Collins *et al*., 1994). Many species within *Clostridium* are capable of polymer degradation and thus extensive research has been done on them regarding

biofuel production from complex biomass (Canganella and Wiegel, 1993; Carreira & Ljungdahl, 1993).

2.7.2 *Thermoanaerobacterium*

Thermoanaerobacterium belongs to the order Thermoanaerobacterales, the class Clostridia and the phylum Firmicutes (Lee *et al*, 1993). *Thermoanaerobacterium* falls into clusters V, VI and VII in the phylogenetic interrelationships of *Clostridium* (Collins *et al*, 1994). The type species for *Thermoanaerobacterium* is *Thermoanaerobacterium thermosulfurigenes* which was reclassified from the genus *Clostridium* in 1993 (Lee *et al*, 2008). Nine validly described species belong to *Thermoanaerobacterium; T. aciditolerans, T. aotearoense, T. saccharolyticum, T. thermosaccharolyticum, T. thermosulfurigenes, T. xylanolyticum, T. fijiensis, T. polysaccharolyticum and T. zeae. Thermoanaerobacterium* species are all strict anaerobic and degrade various carbohydrates to acetate, ethanol, lactate, H_2 and CO_2. Bacteria within this genus reduce thiosulfate to elementary sulfur *(*Lee *et al*, 1993*).*

2.7.3 *Thermoanaerobacter*

Bacteria within the genus *Thermoanaerobacter* used to belong to the genus *Clostridium*. The genus *Thermoanaerobacter* was first described in 1981 by J. Wiegel and L. G. Ljungdahl. The genus falls within the clusters V, VI and VII in phylogenetic interrelationships of *Clostridium* species as does *Thermoanaerobacterium* (Collins *et al*, 1994). The type species is *Thermoanaerobacter ethanolicus*, which has been reported to produce 1.9 mol ethanol mol glucose^{-1}, or the highest yields reported for thermophilic bacteria (Wiegel & Ljungdahl, 1981). Today, the genus consists of 18 named species (Table 3). Most of these species have a broad substrate spectrum and can degrade both hexoses and pentoses. Their main end products are ethanol, acetate, H_2, CO_2 and lactate. Bacteria within the genus are gram-variable and sporulation is variable. Most of them reduce thiosulfate to H_2S (Lee *et al*, 1993).

Table 3 End product formation from glucose by Thermoanaerobacter

			End products			
Organism	Temperature min/opt/max	pH min/opt/max	EtOH	Ac	Lac	H_2+CO_2
T. acetoethylicus	40/>65/80	5.5/XX/8.5	+	+	-	+
T. brockii subsp. brockii	35/65-70/85	5.5/7.5/9.5	+	+	+	+
T. ethanolicus	37/69/78	4.4/5.8-8.5/9.8	+	+	+	+
T. italicus	45/70/78	xx/7.0/xx	+	+	+	+
T. keratinophilus	50/70/80	5.0/7.0/9.0				
T. kivui	xx/66/xx	xx/6.4/xx		+		
T. mathrani	50/70-75/75	4.7/7.0/8.8	+	+	+	+
T. pseudoethanolicus	xx/65/xx		+	+	+	+
T. siderophilus	39/69-71/78	4.8/6.3-6.5/8.2	+		+	+
T. subterraneus	35/65/80	5.5/7.5/9.0	-	+	+	+
T. sulfurigignens	34/63-67/72	4.0/5.0-6.5/8.0	+	+	+	+
T. sulfurophilus	44/55-60/75	4.5/6.8-7.2/8.0	+	+	trace	
T. tengcongensis	50/75/80	5.5/7.0-7.5/9.0	+	+	-	+
T.thermocopriae	47/60/74	6.0/6.5-7.3/8.0	+	+	+	+
T. thermohydrosulfuricus	37/67-69/78	5.5/6.9-7.5/9.2	+	+	+	+
T. uzonensis	32.5/61/69	4.2/7.1/8.9				
T. wiegelii	38/65-68/78	5.5/6.8/7.2	+	+	+	+
T. yonseiensis	50/75/85	4.5/6.5/9.0	+	+	+	+

Of *Thermoanaerobacter* species, *T. acetoethylicus*, *T. thermocopriae,* and *T. yonseiensis* have been reported to produce butyrate whereas formate has only been detected from *T. thermohydrosulfuricus*. *T. etanolicus* has the highest recorded yields of ethanol from glucose, 1.9 mol EtOH mol glc-1 (Carreira *et al*, 1983; Wiegel & Ljungdahl, 1981).

2.7.4 *Caldicellulosiruptor*

The genus was presented in 1994 (Rainey *et al*, 1994). Today, the genus consists of nine extremely thermophilic named species; *C. acetigenus, C. bescii, C. hydrothermalis, C. kristjanssonii, C. kronotskyensis, C. lactoaceticus, C. obsidiansis, C. owensis* and *C. sacchaorolyticus* (Sveinsdottir, M. *et al*, 2009). *C. saccharolyticus* and *C. owensis* have been most thoroughly investigated concerning hydrogen production. *C. saccharolyticus* has been reported to produce 3.60 mol H_2 per mol glucose in a continuous culture (Vrije *et al*, 2007) and *C. owensis* has been reported to produce 3.80 mol H_2 per mol glucose in a continuous culture (Zeidan & van Niel, 2010).

2.7.5 *Thermotoga*

The genus was first presented in 1986. It is a genus of extremely thermophilic, anaerobic bacteria. In fact they grow at the highest reported temperatures for bacteria (Huber *et al*, 1986) The type species is *T. maritima*. Now, the genus consists of nine identified species; *T. elfii, T. hyperogea, T. lettingae, T. maritima, T. naphthophila, T. neapolitana, T. petrophila, T. subterranean and T. thermarum*. Hydrogen production is more thermodynamically favorable at higher temperatures (Kengen et al, 2009), as stated earlier, and this is indeed the case with the genus *Thermotoga*. Within the genus are the species with the highest recorded yields of hydrogen; *T. neapolitana* that produces 3.85 mol H_2 per mol glucose in continuous culture (d'Ippolito *et al*, 2010), and *T. maritima* which has been recorded to produce 4 moles of H_2 per mol glucose in a batch culture (Schroder *et al*, 1994).

3 References

Alexander G & Boyle G. 2004. Introducing Renewable Energy. In Godfrey Boyle (editor), *Renewable energy: Power for að sustainable future.* Second edition, pp. 2-15. UK: University Press, Cambridge.

Almarsdottir AR, Taraceviz A, Gunnarsson I & Orlygsson J. 2010. Hydrogen production from sugars and complex biomass by Clostridium species, AK14, isolated from Icelandic hot spring. *Icelandic Agricultural Sciences* 23:61-71.

Amend & Shock. 2001. Energetics of overall metabolic reactions of themophilic and hyperthermophilic Arhea and Bateria. *FEMS Microbiology Teciews* 25:175-243.

Argun H, Kargi F & Kapdan IK. 2008. Light fermentation of dark fermentation effluent for bio-hydrogen production by different *Rhodobacter* species at different initial volatile fatty acid (VFA) concentration. *International Journal of Hydrogen Energy* 33:7405-7412.

Bai FW, Anderson WA & Moo-Young M. 2008. Ethanol fermentation technologies from sugar and starch feedstocks. *Biotechnology Advances* 26:89-105.

Balat M. 2010. Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review. *Energy Conversion and Management* 52:858-875.

Balusu R, Paduru RMR, Seenayya G & Reddy G. 2004. Production of ethanol from cellulosic biomass by *Clostridium thermocellum* SS19 in submerged fermentation - screening of nutrients using plackett-burman design. *Applied Biochemistry and Biotechnology* 117: 133-141.

Balusu,R, Paduru RR, Kuravi SK, Seenayya G. & Reddy G. 2005. Optimization of critical medium components using response surface methodology for ethanol production from cellulosic biomass by *Clostridium thermocellum* SS19. *Process Biochemistry* 40:3025-3030.

Ben Bassat A, Lamed R, & Zeikus JG. 1981. Ethanol-production by thermophilic bacteria - metabolic control of end product formation in thermoanaerobium-brockii. *Journal of Bacteriology* 146(1):192-199.

Bergquist PL, Gibbs MD, Morris DD, Teo' VS, Jsaul DJ & Morgan HW 1999. Molecular diversity of thermophilic cellulolytic and hemicellulolytic bacteria. *FEMS Microbiology Ecology* 28:99-110.

Bornscheuer U and Kazlauskas RJ. 2006. *Hydrolases in Organic Synthesis: Regio- and Stereoselective Biotransformations* (second edition). WILEY-VCH Verlag GmbH and Co. KGaA: Weinheim.

Brennan A H, Hoagland W & Schell DJ. 1986. High temperature acid hydrolysis of biomass using an engineering-scale plug flow reactor: result of low solids testing. *Biotechnology and Bioengineering Symposium* 17:53-70.

Brigham JS, Adney WS & Himmel ME. 1996. Hemicellulases: diversity and applications. Handbook on bioethanol production and utilization (p. 119-141). Washington, DC: Taylor & Francis.

Brock TD. 1986. Introduction: an overview of the thermophiles, In: T.D. Brock (editor) *Thermophiles: General Molecular and Applied Microbiology*, pp. 1-16. New York, USA: John Wiley and Sons.

Brownell HH and Saddler JN. 1984. Steam explosion pretreatment for enzymatic hydrolysis. *Biotechnology and Bioengineering Symposium* 14:55–68.

Cahela DR, Lee YY & Chambers RP. 1983. Modeling of percolation process in hemicellulose hydrolysis. *Biotechnology and Bioengineering*. 25:3-17.

Canagella F & Wiegel J. 1993. The potential of thermophilic Clostridia in biothechnology. In D.R. Woods (editor) *the Clostridia and Biothechnology*, pp. 391-429. Stoneham, MA, USA: Butterworths Publishers.

Carreira LH & Ljungdahl LG. 1993. Production of ethanol from biomass using anaerobic thermophilic bacteria, In D.L. Wise (editor) *Liquid fuel development*, pp. 1-28. Boca Raton, FI, USA: CRC Press.

Carreira LH, Wiegel J & Ljungdahl LG. 1983. Production of ethanol from bio-polymers by anaerobic, thermophilic, and extreme thermophilic bacteria. I. Regulation of carbohydrate utilization in mutants of Thermoanaerobacter ethanolicus. *Biotechnology and Bioengineering* 13:183-191.

Carreira LH, Wiegel J & Ljungdahl LG. 1983. Production of ethanol from bio-polymers by anaerobic, thermophilic, and extreme thermophilic bacteria. I. Regulation of carbohydrate utilization in mutants of Thermoanaerobacter ethanolicus. *Biotechnology and Bioengineering* 13:183-191.

Cavallo AJ. 2004. Hubbert's Petroleum Production Model: An Evaluation and Implications for World Oil Production Forecasts. *Natural Resources Research* 13(4):211-221.

Chang T & Yao S. 2011. Thermophilic, lignocellulolytic bacteria for ethanol production: Current state and perspectives. *Applied Microbiology and Biotechnology* 92(1):13-27. doi:10.1007/s00253-011-3456-3

Chang VSV & Holtzapple MTM. 2000. Fundamental factors affecting biomass enzymatic reactivity. *Applied Biochemistry and Biotechnology*. 84-86(0273-2289, 0273-2289), 5-37.

Christian V, Shrivastava R, Shukla D, Modi HA & Vyas BRM. 2005. Degradation of xenobiotic compounds by lignin-degrading white-rot fungi: Enzymology and mechanisms involved. *Indian journal of Experimental Biology* 43:301-312.

Clark TA & Mackie KL. 1987. Steam explosion of the soft-wood Pinus radiata with sulphur dioxide addition. 1. Process optimization. *Journal of Wood Chemistry and Technology* 7(3):373-403.

Collins MD, Lawson PA, Willems A, Cordoba JJ, Fernandezgarayzabal J, Garcia P, Cai J, Hippe H & Farrow JAE. 1994. The phylogeny of the genus *Clostridium* - proposal of 5 new genera and 11 new species combinations. *International Journal of Systematic Bacteriology* 44(4):812-826.

Converse AOK, Warteng IKm Grethlein HE & Ooshima H. 1989. Kinetics of thermochemical pretreatment of lignocellulosic materials. *Applied Biochemistry and Biotechnology* 20/21:63-78.

d'Ippolito G, Dipasquale L, Vella FM, Romano I, Gambacorta A, Cutignano A & Fontana A. 2010. Hydrogen metabolism in the extreme thermophile *Thermotoga neapolitana*. *International Journal of Hydrogen Energy* 35(6):2290-2295. doi:10.1016/j.ijhydene.2009.12.044

da Costa Sousa L, Chundawat SP, Balan V & Dale BE. 2009. 'Cradle-to-grave' assessment of existing lignocellulose pretreatment technologies. *Current Opinion in Biotechnology* 20(3):339-347. doi:10.1016/j.copbio.2009.05.003

Dien BS, Cotta MA & Jeffries TW. 2003. Bacteria engineered for fuel ethanol production: current status. *Appl Microbiol Biotechnol* 63:258-266.

Duchane DV. 1994. Status of the United States hot dry rock technology development program. *Geothermal Technology* 19(1/2):12–30.

Duff SJB & Murray WD. 1996. Bioconversion of forest products industry waste cellulosics to fuel ethanol: a review. *Bioresource Technology* 55:1-33.

EIA (Energy Information Administration). 2009. Annual US Field Production of Crude Oil, 1859–2008. 20. mars retrieved from http://www.eia.gov/dnav/pet/hist/LeafHandler.ashx?n=PET&s=MCRFPUS1&f=A

EIA (U.S. Energy Information Administration). (21. january 2009). International Energy Annual (IEA) – *long term historical international energy statistics*. 9. mars 2012 retrieved from http://205.254.135.7/iea/

EIA (U.S. Energy Information Administration). 2011. *International Energy Outlook 2011*. 9. mars 2012 retrieved from http://www.eia.gov/totalenergy/reports.cfm?t=182

EIA (U.S. Energy Information Administration). n.d. *International Energy statistics*. 9. mars 2012 retrieved from http://www.eia.gov/cfapps/ipdbproject/iedindex3.cfm?tid=44&pid=44&aid=1&cid=ww,&syid=2003&eyid=2008&unit=QBTU

Environmental Literacy Council. 2008a. *Biomass Energy.* 15. mars 2012 retrieved from: http://www.enviroliteracy.org/article.php/10.html

Environmental Literacy Council. 2008b. *Energy*. 15. mars 2012 retrieved from: http://www.enviroliteracy.org/category.php/4.html

Environmental Literacy Council. 2008c. *Geothermal energy.* 15. mars 2012 retrieved from: http://www.enviroliteracy.org/article.php?id=54&

Environmental Literacy Council. 2008d. *Hydroelectric power.* 15. mars 2012 retrieved from: http://www.enviroliteracy.org/article.php/59.php

Eriksen NT Nielsen TM and Iversen N. 2008. Hydrogen production in anaerobic and microaerobic *Thermotoga neapolitana*. *Biotechnology Letters* 30(1):103-109.

Esteghlalian A, Hashimoto AG, Fenske JJ & Penner MH. 1997. Modeling and optimization of the dilute-sulfuric-acid pretreatment of corn stover, poplar and switchgrass. *Bioresource Technology* 59:129-136.

Faber K. 2004. *Biotransformatins in Organic Chemistry* (fifth edition). Germany: Springar-Verlag.

Fackler K, Gradinger C, Hinterstoisser B, Messner K & Schwanninger M. 2006. Lignin degradation by white rot fungi on spruce wood shavings during short-time solid-state fermentations monitored by near infrared spectroscopy. *Enzyme and Microbial Technology* 39(7):1476-1483. doi:10.1016/j.enzmictec.2006.03.043

Fan LT, Gharpuray MM & Lee YH. 1987. *Cellulose Hydrolysis Biotechnology Monographs*. Berlin: Springer Verlag.

Fernández JR, Abanades JC, Murillo R & Grasa G. 2012. Conceptual design of a hydrogen production process from natural gas with CO_2 capture using a Ca–Cu chemical loop. *International Journal of Greenhouse Gas Control* 6(0):126-141. doi:10.1016/j.ijggc.2011.11.014

Galletti, G. C., & Bocchini, P. (1995). "Pyrolysis/gas chromatography/mass spectrometry of lignocellulose. *Rapid Communications in Mass Spectrometry* 9:815-826.

Georgieva TI & Ahring BK. (2007). Evaluation of continuous ethanol fermentation of dilute-acid corn stover hydrolysate using thermophilic anaerobic bacterium *Thermoanaerobacter* BG1L1. *Appl. Microbiol. Biotechnol*. 77:61–68.

Georgieva TI, Mikkelsen MJ & Ahring BK. 2008a. Ethanol production from wetexploded wheat straw hydrolysate by thermophilic anaerobic bacterium *Thermoanaerobacter* BG1L1 in a continuous immobilized reactor. *Applied Biochemistry and Biotechnology* 145:99-110.

Georgieva TI, Mikkelsen MJ & Ahring BK. 2008b. High ethanol tolerance of the thermophilic anaerobic ethanol producer *Thermoanaerobacter* BG1L1. *Central European Journal of Biology* 2:364-377.

Gibbs MD, Saul DJ, Luthi E & Bergquist PL. 1992. The β-mannanases from "*Caldocellum saccharolyticum*" is part of a multidomain en- zyme. *Applied Environmental Microbiology* 58:3864-3867.

Glazer AN & Nikaido H. 2007. *Microbial Biotechnology: Fundamentals of Applied Microbiology,* Second Edition. New York: W:H: Freeman and Company.

Goettemoeller J & Goettemoeller A. 2007. *Sustainable Ethanol: Biofuels, Biorefineries, Cellulosic Biomass, Flex-Fuel Vehicles, and Sustainable Farming for Energy Independence*. Maryville, Missouri: Prairie Oak Publishing.

Grey KA, Zhao L & Emptage M. 2006. Bioethanol. *Current Opinion in Chemical Biology* (10),141-146.

Hallenbeck PC, Ghosh D, Skonieczny MT & Yargeau V. 2009. Microbiological and engineering aspects of biohydrogen production. *Indian Journal of Microbiology* 49:48-59.

Hallenbeck PC, Kochian KV, Weissman JC & Benemann JR. 1978. Solar energy conversion with hydrogen producing cultures of the blue-green alga, *Anabaena cylidrical*. *Biotechnology and Bioengineering* 8:283-297.

Hemmingsen E 2010. At the base of Hubbert´s peak: Grounding the debate on petroleum scarcity. *Geoforum* 41:(4):531-540.

Hendriks ATWM & Zeeman G. 2008. Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource Technology* 100:10–18.

Hsu TA, Ladisch MR & Tsao GT. 1980. Alcohol from cellulose. *Chemical Technology* 10(5):315-319.

Huber R, Langworthy TA, Konig H, Thomm M, Woese CR, Sleytr UB & Stetter KO. 1986. *Thermotoga maritima* sp. nov., represents a new genus of unique extremely thermophilic eubacteria growing up to 90-degrees-C. *Archives of Microbiology* 144(4):324-333.

Jeffries TW. 2006. Engineering yeasts for xylose metabolism. *Current Opinion in Biotechnology* 17: 320–326

Joachimsthal EL & Rogers PL. 2000. Characterization of a high productivity recombinant strain of *Zymomonas mobilis* for ethanol production from glucose/xylose mixtures. *Applied Biochemistry and Biotechnology* 84–86:343–356.

Kengen SWM, Goorissen HP, Verhaart M, van Niel EWJ, Claassen PAM & Stams AJM. 2009. Biological hydrogen production by anaerobic microorganisms. In W. Soetaert & E.J. Vandamme (editors) *Biofuels,* pp. 197-221. Chichester: John Wiley & Sons.

Kosaric N, Pieper HJ, Senn T & Valdar-Sukan F. 2001. *The Biothechnology of Ethanol: Classical and Future Applications*. Weinheim: WILEY-VCH Verlag GmbH

Koskinen PEP, Lay CH, Puhakka JA, Lin PJ, Wu SY, Orlygsson J & Lin CH. 2008. High efficiency hydrogen production by an anaerobic thermophilic enrichment culture from Icelandic hot spring. *Biotechnology and Bioengineering* 101:665-678.

Kotay SM & Das D. 2008. Biohydrogen as a renewable energy source - Prospects and potentials. *International Journal of Hydrogen Energy* 33.:258-263.

Kristjansson JK & Alfredsson GA. 1986. Life in Icelandic Hot Sprins, *Natturufraedingurinn* 56: 49-68.

Kumaresan Jacob, Narain Jai P & Sathiakumar Nalini. 2011. Climate change and health in South East Asia. *International journal of Climate Change Strategies and Management*. 3(2):200-208. doi: 10.1108/17568691111129020

Lamed R & Zeikus JG. 1980a. Ethanol-production by thermophilic bacteria - relationship between fermentation product yields of and catabolic enzyme-activities in *Clostridium thermocellum* and *Thermoanaerobium brockii*. *Journal of Bacteriology* 144(2):569-578.

Lamed R & Zeikus JG. 1980b. Glucose fermentation pathway of thermoanaerobium-brockii. *Journal of Bacteriology* 141(3):1251-1257.

Lau MW, Dale BE & Balan, V. 2008. Ethanolic fermentation of hydrolysates from ammonia fiber expansion (AFEX) treated corn stover and distillers grain without detoxification and external nutrient supplementation. *Biotechnology and Bioengineering* 99:529–539.

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

Lee J. 1997. Biological conversion of lignocellulosic biomass to ethanol. *Journal of Biotechnology* 56 (1):1-24.

Lee YE, Jain MK, Lee CY, Lowe SE & Zeikus JG. 1993. Taxonomic distinction of saccharolytic thermophilic anaerobes: Description of *Thermoanaerobacterium xylanolyticum* gen. nov., sp. nov., and *Thermoanaerobacterium saccharolyticum* gen. nov., sp. nov.; reclassification of *Thermoanaerobium brockii, Clostridium thermosulfurogenes,* and *Clostridium thermohydrosulfuricum* E100-69 as *Thermoanaerobacter brockii* comb. nov., *Thermoanaerobacterium thermosulfurigenes* comb. nov., and *Thermoanaerobacter thermohydrosulfuricus* comb. nov., respectively; and transfer of *Clostridium thermohydrosulfuricum* 39E to *Thermoanaerobacter ethanolicus. International Journal of Systematic Bacteriology* 43(1):41-51.

Lehninger AL, Nelson DL & Cox MM. 2005. *Lehninger principles of biochemistry, Volume 1, fourth edition*. Útgáfustaður: W. H. Freeman.

Leroy RL. 1983. Industrial water electrolysis - present and future. *International Hournal of Hydrogen Energy* 8:401-417.

Levin DB, Islam R, Cicek N & Sparling R. 2006. Hydrogen production by *Clostridium thermocellum* 27405 from cellulosic biomass substrates. *International Journal of Hydrogen En*ergy 31(11):1496-1503. doi:10.1016/j.ijhydene.2006.06.015

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

Lin C-W, Wu C-H, Tran D-T, Shih M-C, Li W-H & Wu C-F. 2010. Mixed culture fermentation from lignocellulosic materials using thermophilic lignocellulose degrading anaerobes. *Process Biochemistry* 46:489-493.

Liu C, Dong H Zhong J, Ryu D & Bao J. 2010. Sorbitol production using recombinanat *Zymomonas mobilis* strain. *Journal of Biotechnol* 148:105-112.

Liu H, Zhang T & Fang HHP. 2003. Thermophilic H2 production from a cellulose-containing wastewater. *Biotechnology Letters* 25:365-369.

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

Lynd LR, Weimer PJ, van Zyl WH & Pretorius IS. 2002. Microbial Cellulose Utilization: Fundamentals and Biotechnology. *Microbiology and molicular biology reviews* 66:506-577.

Madigan MT, Martinko JM & Parker J. 2003. *Brock Biology of Microorganisms* (10th ed.) upper Saddle Rever: Prentice Hall.

Mauna Loa Observatory. 2011. CO_2. 28. Mars 2012 retrieved from http://scrippsco2.ucsd.edu/data/atmospheric_co2.html

McMurry J & Simanek E. 2007. Fundamentals of Organic Chemistry, 6.ed. South Melbourne: Cengage Learning.

McNaught A D & Wilkinson A. 2006. *Compendium of Chemical Terminology*, (2nd edition). (the "Gold Book"). doi:10.1351/goldbook

Miyamoto K, Hallenbeck PC & Benemann JR. 1979. Solar energy conversion by nitrogen limited cultures of *Anabaena cylindrica*. *Ferment Technol* 57:287-293.

Mok WS-L & Antal MJ Jr. 1992. Uncatalyzed solvolysis of whole biomass hemicellulose by hot compressed liquid water. *Industrial Engineering Chemistry Research* 31:1157–1161.

Mok WS-L & Antal MJ. Jr. 1994. Biomass fractionation by hot compressed liquid water. In A.V. Bridgewater (editor), *Advances in Thermochemical Biomass*

Conversion, volume 2, pp. 1572–1582. New York: Blackie Academic & Professional Publishers.

Monserrate E, Leschine SB & Canale-Parola E. (2001). *Clostridium hungatei* sp. nov., a mesophillic, N_2-fixing cellulolytic bacterium isolated from soil. *International Journal of Systematic and Evolutionary Mycrobiology* 51:123–32.

Mosier NS, Wyman CE, Dale BE, Elander RT, Lee YY, Holtzapple MT & Ladisch MR. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology* 96:673–686.

Munro SA, Zinder SH & Walker LP. 2009. The fermentation stoichiometry of *Thermotoga neapolitana* and influence of temperature, oxygen, and pH on hydrogen production. *Biotechnology Progress* 25(4):1035-1042.

Nakashimada Y, Rachman MA, Kakizono T & Nishio N. 2002. Hydrogen production of *Enterobacter aerogenes* altered by extracellular and intracellular redox states. *International Journal of Hydrogen Energy* 27(11–12):1399-1405. doi:10.1016/S0360-3199(02)00128-3

Nardon L & Aten K. 2008. Beyond a better mousetrap: A cultural analysis of the adoption of ethanol in Brazil. *Journal of World Business* 43:261-273.

Nass LLP, Pereira AA & Ellis D. 2007. Biofuels in Brazil: An overview. *Crop Science* 47:2228-2237.

Nguyen TAD, Kim JP, Kim MS, Oh YK & Sim SJ. 2008a. Optimization of hydrogen production by hyperthermophilic eubacteria, *Thermotoga maritima* and *Thermotoga neapolitana* in batch fermentation. *International Journal of Hydrogen Energy*, 33(5):1483-1488.

Nguyen TD, Han SJ, Kim JP, Kim MS & Sim SJ. 2010a. Hydrogen production of the hyperthermophilic eubacterium, *Thermotoga neapolitana* under N-2 sparging condition. *Bioresource Technology* 101(1):S38-S41.

Nguyen TD, Han SJ, Kim JP, Kim MS, Oh YK & Sim SJ. 2008. Hydrogen production by the hyperthermophilic eubacterium, *Thermotoga neapolitana*, using cellulose pretreated by ionic liquid. *International Journal of Hydrogen Energy* 33(19):5161-5168. doi:10.1016/j.ijhydene.2008.05.019

Nguyen TD, Kim K-R, Nguyen M-T, Kim MS, Kim D & Sim SJ. 2010c. Enhancement of fermentative hydrogen production from green algal biomass by *Thermotoga neaolitana* by various pretreatment methods. *International Journal of Hydrogen Energy* 35(23):13035-13040.

Nguyen TD, Kim K, Kim MS & Sim SJ. 2010b. Thermophilic hydrogen fermentation from korean rice straw by *Thermotoga neapolitana*. *International Journal of Hydrogen Energy* 35(24):13392-13398.

Ni M, Leung DY, Leung MK & Sumathy K. 2006. An overview of hydrogen production from biomass. *Fuel Processing Technology* 87:461-472.

Noyes PD, McElwee MK, Miller HD, Clark BW, Van Tiem LA,Walcott KC, Erwin KN & Levin ED. 2009. The toxicology of climate change: environmental

contaminants in a warming world. *Environment International* 35(6):971-986. Doi: http://dx.doi.org/10.1016/j.envint.2009.02.006

NREL (National Renewable Energy Laboratory). 2010. Energy efficiency and Renewable Energy: 2010 Renewable Energy Data Book. 4. april retrieved from http://www.nrel.gov/analysis/pdfs/51680.pdf

Oh Y, Seol E, Jung Rae Kim JK & Park S. 2003. Fermentative biohydrogen production by a new chemoheterotrophic bacterium *Citrobacter* sp. Y19. Int. *Journal of Hydrogen Energy* 28:1353 – 1359.

Panwar NL, Kaushik SC & Kothari S. 2010. Role of renewable energy sources in environmental protection: A review. *Renewable and Sustainable Energy Reviews* 15:1513-1524.

Perry JJ, Staley JT & Lory S. 2002. *Microbial Life*. Sunderland, Massachusetts: Sinauer Associates, Inc.

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

Rittmann BE. 2008. Opportunities for renewable bioenergy using microorganisms, *Biotechnology Bioengineering* 100:203-212.

Rogers PL, Lee KJ, Skotnicki ML & Tribe DE. 1982. Ethanol production by Zymomonas Mobilis. *Advances in Biochemical Engineering* 23:37-84.

Salo S, Niemelä S, Elomaa M and Lindberg JJ. 1989. Identification of lignins by pyrolysis methods. *Holzforschung* 43(4):257-260.

Sanchez RG, Karhumaa K, Fonseca C, Nogue VS, Almeida JRM, Larsson CU, Bengtsson O, Bettinga M, Hahn-Hagerdal B & Gorwa-Grauslund MF. 2010. Improved xylose and arabinose utilization by an industrial recombinant Saccharomyces cerevisae strain using evolutionary engineering. *Biotechnology for Biofuels* 3:1-11.

Schicho RN, Ma K, Adams MWW & Kelly RM. (1993). Bioenergetics of sulfur-reduction in the hyperthermophilic archaeon Pyrococcus furiosus, *Journal of Bacteriology* 175(6):1823-1830.

Schroder C, Selig M & Schonheit P. 1994. Glucose fermentation to acetate, Co2 and H-2 in the anaerobic hyperthermophilic eubacterium *Thermotoga maritima* - involvement of the Embden Meyerhof pathway. *Archives of Microbiology* 161(6):460-470.

Shu-Wei Chang, Wei-Hsin Chang, Maw-Rong Lee, Tzung-Jie Yang, Nu-Yi Yu, Chin-Shuh Chen, & Jei-Fu Shaw. 2010. Simultaneous Production of Trehalose, Bioethanol, and High-Protein Product from Rice by an Enzymatic Process. *Journal of Agricultural and Food Chemistry* 58 (5):2908-2914.

Sivers M V & Zacchi G. 1995. A techno-economical comparison of three processes for the production of ethanol from pine. *Bioresource Technology* 51:43-52.

Soboh B, Linder D & Hedderich R. 2004. A multisubunit membrane-bound [NiFe] hydrogenase and an NADH-dependent Fe-only hydrogenase in the fermenting bacterium *Thermoanaerobacter tengcongensis*. *Microbiology* 150(7):2451-2461.

Solar Energy Industries Association, (n.d.) About Solar Energy. 16. mars 2012 retrieved from http://www.seia.org/cs/about_solar_energy

Srinivasan S. 2008. The food v. fuel debate: A nuanced view of incentive structures. *Renewable Energy* 34(4):950-954.

Stephanopoulos G. 2007. Challenges in engineering microbes for biofuels production. *Science* 315:801-804.

Stevnebø A, Sahlström, S & Svihus B. 2006. Starch structure and degree of starch hydrolysis of small and large strach granules from barley varieties with varying amylose content. *Animal Feed Science and Technology* 130 (1-2): 23-38.

Sun Y & Cheng JY. 2002. Hydrolysis of lignocellulosic materials for ethanol production: A review. *Bioresource Technology* 83(1):1-11.

Sveinsdottir M, Baldursson SRB & Orlygsson J. 2009. Ethanol production from monosugars and lignocellulosic biomass by thermophilic bacteria isolated from icelandic hot springs. *Icelandic Agricultural Sciences* 22:45-58.

Swings, J and De Ley. 1977. The biology of *Zymomonas*. *American J. Bacteriol. Rev*. 41:1-46.

Tassinari T, Macy C & Spano L. 1980. Energy requirements and process design considerations in compression-milling pretreatment of cellulosic wastes for enzymatic hydrolysis. *Biotechnology and Bioengineering* 22:1689–1705.

Te'o VS, Saul DJ & Bergquist PL. 1995. CelA, another gene coding for a multidomain cellulases from the extreme thermophile *Caldocellum saccharolyticum*. *Applied Microbiology and Biotechnology* 43(2):291-296.

Turner JA. 2004. Sustainable hydrogen production. *Science*, 305(5686), 972-974.

U.S Department of Energy. 2005a. *History of hydropower*. 15. mars 2012 retrieved from: http://www1.eere.energy.gov/wind andhydrohystory.html

U.S Department of Energy. 2005b. *How hydropower works, Energy efficiency & renewable energy*. 15. mars 2012 retrieved from: http://www1.eere.energy.gov/windandhydro/hydro_how.html

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

van Maris AJ, Winkler AA, Kuyper M, de Laat WT, van Dijken JP & Pronk JT. 2007. Development of efficient xylose fermentation in *Saccharomyces cerevisae*: xylose isomerase as a key component. Advaces in Biochemical Engineering/ Biotechnology 108:179-204.

van Niel EWJ, Budde MAW, de Haas GG, van der Wal FJ, Claasen PAM & Stams AJM. 2002. Distinctive properties of high hydrogen producing extreme thermophiles, *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii. International Journal of Hydrogen Energy* 27(11-12):1391-1398.

van Niel EWJ, Claassen PAM & Stams AJM. 2003. Substrate and product inhibition of hydrogen production by the extreme thermophile, *Caldicellulosiruptor saccharolyticus*. *Biotechnology and Bioengineering* 81(3):255-262. doi:10.1002/bit.10463

Vrije G.JDe, Mars AE, Budde MAW, Lai MH, Dijkema C, Waard PDe & Classen PAM. 2007. Glycolytic pathway and hydrogen yield studies of the extreme thermophile *Caldicellulosiruptor saccharolyticus. Applied Microbiology and Biotechnology* 74:1358-1367.

Wang DIC, Avgerinos GC, Biocic I, Wang SD & Fang HY 1983. Ethanol from cellulosic biomass. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* 300:323–333.

Weil JR, Dien B, Bothast RJ, Hendrickson R, Moiser NS & Ladisch MR. 2002. Removal of fermentation inhibitors formed during pretreatment of biomass by polymeric adsorbents. *Industrial & Engineering Chemistry Research* 41:6132–6138

Wiegel J & Lungdahl LG. 1981. Thermoanaerobacter ethanolicus gen. nov., spec. nov., a new extreme thermophilic, anaerobic bacterium. *Archives of Microbiology* 128:343-348.

Wiegel J, Carreira LH, Mothershed CP & Puls, J. 1983. Production of ethanol from biopolymers by anaerobic, thermophilic, and extreme thermophilic bacteria. II. *Thermoanaerobacter ethanolicus* JW200 and its mutants in batch cultures and resting cell experiments. *Biotechnology and Bioengineering* 13:193-205.

Wisselink HW, Toirkens MJ, Wu Q, Pronk JT & van Maris AJA. 2008. Novel evolutionary engineering approach for accelerated utilization of glucose, xylose and arabinose mixtures by engineered *Saccharomyces cerevisiae* strains. *Applied and Environmental Microbiology* 75:907-914.

Yanase H, Sato D, Yamamoto K, Matsuda S, Yamamoto S & Okamoto K. 2007. Genetic engineering of *Zymobacter palmae* for production of ethanol from xylose. *Applied and Environmental Microbiology* 73:2592-2599.

Yetis M, Gunduz U, Erogul I, Yucel M & Turker L. 2000. Photoproduction of hydrogen from sugar refinery wastewater by *Rhodabacter sphaeroides* O.U. 001. *International Journal of Hydrogen Energy* 25(11):1035-1041.

Zeidan AA & van Niel EWJ. 2010. A quantitative analysis of hydrogen production efficiency of the extreme thermophile *Caldicellulosiruptor owensis* OLT.

International Journal of Hydrogen Energy 35(3)1128-1137. doi:10.1016/j.ijhydene.2009.11.082

Zeng K & Zhang D. 2009. Recent progress in alkaline water electrolysis for hydrogen production and applications, *Progress in Energy and Combustion Science* 36:307-326.

Zheng YZ, Lin HM & Tsao GT. 1998. Pretreatment of cellulose hydrolysis by carbon dioxide explosion. *Biotechnology Progress* 14:890-896.

4 Manuscript I

Ethanol production from sugars and complex biomass by *Thermoanaerobacter* AK_5: The effect of electron scavenging systems on end product formation

Ethanol production from sugars and complex biomass by *Thermoanaerobacter* AK_5: The effect of electron scavenging systems on end product formation

Hronn BRYNJARSDOTTIR, Johann ORLYGSSON

University of Akureyri, Faculty of Natural Resource Sciences

Mailing address: Borgir, Nordurslod, 600 Akureyri, Iceland

Address: Faculty of Natural Resource Science, University of Akureyri, Borgir, Nordurslod, 600 Akureyri, Iceland. Tel: +354 4608511. Fax: + 354 4609889.

E-mail address: jorlygs@unak.is

Abstract

The ethanol production capacity from sugars and lignocellulosic biomass hydrolysates by *Thermoanaerobacter* strain AK_5 was studied in batch cultures. The strain converts various carbohydrates to acetate, ethanol, hydrogen and carbon dioxide. Maximum ethanol yields on glucose and xylose were 1.70 and 1.35 mol/mol sugar, respectively. Increased initial glucose concentration, up to 30 mM, increased the end product formation but levelled off at higher concentrations. Cultivation of the strain on glucose with decreasing liquid-gas ratios resulted in a shift to more acetate and less ethanol. End product formation from glucose was further manipulated by adding extracellular electron acceptor (thiosulfate) or by using a co-culture of hydrogenotrophic methanogen. In both cases, the hydrogen scavenging systems resulted in a dramatic shift from ethanol to acetate. Ethanol production was investigated by growing on 4.5 g L^{-1} of complex biomass hydrolysates of grass, hemp, wheat straw, newspaper and cellulose that was pretreated with acid (0.50% H_2SO_4), alkali (0.50% NaOH) and controls without acid/alkali treatment, followed by incubation with the enzymes Celluclast® and Novozymes 188 (0.1 mL g^{-1} dw; 70 and 25 U g^{-1} of Celluclast and Novozyme 188, respectively). The highest ethanol yields (27.5 mM; 5.5 mM g^{-1} biomass) were obtained on cellulose but lowest on hemp leafs (5.1 mM; 0.8 mM g^{-1}). Chemical pretreatment increased ethanol yields substantially from lignocellulosic biomass but not from cellulose. Most pronounce increase was on straw hydrolysates where ethanol production increased from 5.5 mM to 15.2 mM on alkali pretreated biomass. Ethanol yields were increased from 5.5 mmol to 7.7 mmol g^{-1} on cellulose by decreasing the hydrolysate concentration to 2.25 g L^{-1} because of incomplete glucose degradation in the higher loaded system.

Keywords: Ethanol, *Thermoanaerobacter*, lignocellulose, hydrolysates, electron scavenging system

Introduction

Lignocellulosic biomass is composed of cellulose, hemicellulose and lignin and is very complex compared to starch and sugars, which are the substrates that are responsible for more than 95% of total world ethanol production today (Renewable Fuel Association, 2011). The main reason for the unsuccessful implementation of more complex biomass for fuel production lies in their complex structure and the pretreatments needed for their degredation to hexoses and pentoses (Gírio et al., 2010).

Another bottleneck for ethanol production from lignocellulose is the lack of suitable microorganisms with broad substrate spectrum. The two best known ethanol producing microorganisms are *Saccharomyces cerevisae* and *Zymomonas mobilis*. Both have very narrow substrate spectrum and may not be suitable for ethanol production from more complex substrates. Therefore, increased interest has been on the use of thermophilic bacteria with broad substrate range and high yields. After the oil crisis in the 1980's publications on thermopilic ethanol producing bacteria started to appear in literature (Wiegel, 1980; Wiegel and Ljungdahl, 1981). Thermophilic bacteria within the genera of *Thermoanaerobacterium*, *Thermoanaerobacter* and *Clostridium* have been investigated and showed good ethanol yields and fast growth rates (Lacis and Lawford, 1988; Orlygsson et al., 2010; Sveinsdottir et al., 2009). There are several advantages to 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. 1.5 to 1.9 mol-EtOH mol-hexose^{-1} degraded but the theoretical maximum yield is 2.0 mol-EtOH mol-hexose^{-1} (Avci et al., 2006; Carreira et al., 1983; Koskinen et al., 2008; Lacis and Lawford, 1988; Lovitt et al., 1988; Sveinsdottir et al., 2009; Wiegel and Ljungdahl, 1981).

Relatively few studies are on ethanol yields from hydrolysates made from lignocellulosic biomass. The present study focuses on a newly isolated thermophilic bacterium, *Thermoanaerobacter* AK_5. Bacteria within this genus seem to be very efficient ethanol producers both from simple sugars (Avci et al. 2006; Carreira et al. 1983; Fardeau et al. 1996; Georgieva et al. 2008; Wiegel & Ljungdahl, 1981) as well as from complex biomass (Avci et al., 2006; Georgieva et al., 2007; Georgieva et al., 2008; Rani et al., 1998; Sommer et al., 2004; Wiegel et al., 1983). Bacteria within the genus are Gram variable rods with broad substrate spectrum (mostly sugars) and produce ethanol, acetate, lactate, hydrogen and carbon dioxide during fermentation (Carlier et al., 2006; Lee et al., 1993).

In this paper, we describe the physiological characteristics of *Thermoanaerobacter* AK_5, isolated from Icelandic hot spring with the main focus on the ethanol production capacity.

Materials and methods

Medium

The medium (per liter) consisted of: NH_4Cl 0.3 g, NaCl 0.3 g, $CaCl_2$ 0.11 g, $MgCl_2$ x $6H_2O$ 0.1 g, yeast extract 2.0 g, resazurin 1 mg, trace element solution 1 ml, vitamin solution 1 ml and $NaHCO_3$ 0.8 g. Phosphate buffers were also used where 1 M stock solutions of NaH_2PO_4 and Na_2HPO_4 were made and added to the media to give a buffer capacity of 30 mM. The vitamin solution was according to DSM141. The trace element was as described earlier by Örlygsson and Baldursson (2007). The medium was prepared by adding the phosphate buffer, yeast extract and resazurin to distilled water, which was then boiled for 5-10 min and cooled while flushing with nitrogen. The mixture was then transferred to cultivation bottles and autoclaved for 60 minutes. All other components of the medium were added separately through filter-sterilized solutions. The gas phase in all experiments consisted of pure nitrogen except for the isolation of the methanogen when a mixture of H_2 and CO_2 was used (80/20). All

experiments were performed at 65°C at pH 7.0 without agitation. The inoculum volume was 2% in all experiments. All experiments were done in duplicates.

Isolation of strains

Strain AK_5 was isolated on glucose (20 mM) from a hot spring (64°C, pH 6.7) in Grensdalur in SW Iceland. Sample was collected by using an extended pole with grip arms placed at the end. Sterile serum bottles (117.5 mL) were fixed at the end, opened and completely filled with geothermal mud/liquid sample and closed with butyl rubber and aluminium caps and transferred to the laboratory. Pure culture was obtained by repeated enrichment on glucose followed by end point dilutions and isolations of colonies from agar plates (Orlygsson and Baldursson, 2007). A hydrogenotrophic methanogenic strain (HT) was also isolated on BM medium but instead of using nitrogen as gas phase a mixture of H_2/CO_2 (80/20) was used. Again pure cultures were obtained by repeated enrichments and end point dilutions. For the final dilution series, antibiotics (penicillin; 54 µg mL^{-1} and streptomycin; 80 µg mL^{-1}) were used to exclude heterotrophic bacterial growth.

Optimum growth conditions and kinetics of glucose degradation

To determine the strain's optimum pH for growth the initial pH was set to various levels in the range of 3.0 to 9.0 with increments of 1.0 pH unit. The experimental bottles were supplemented with acid (HCl) and alkali (NaOH) to set the pH accordingly. To determine the optimum temperature for growth the incubation temperature varied from 35°C to 80°C. For the pH optimum the strain was cultivated at 65°C and for the temperature optimum the pH was 7.0. Optimal pH and temperature were used in all experiments performed. Experiments were done in 117.5 mL serum bottles with 50 mL liquid medium. In one set of experiment, kinetics of the strain was done on glucose (20 mM) were gaseous (0.2 mL) and liquid (1.0 mL) samples were withdrawn at certain time intervals and glucose, end products, growth (optical density) as well as pH were analysed.

Phylogenetic analysis

Partial 16S rRNA analysis of 716 nucleotide long sequence was done according to Orlygsson and Baldurson (2007) and references therein. Sequences from 16S rRNA analysis were compared to sequences in the NCBI database using the nucleotide-nucleotide BLAST (BLASTN) tool. The most similar sequences were aligned with the sequencing results in the programs BioEdit (Hall, 1999) and CLUSTAL_X (Thompson et al., 1997). Finally, the trees were displayed with the program TreeView. *Caloramator viterbensis* was used as an outgroup.

Effect of initial glucose concentration on end product formation

The effect of glucose concentration on strain AK_5, by varying the concentration from 5 to 100 mM, was tested. Control samples did not contain glucose. Glucose, hydrogen, acetate 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 60 mL liquid medium. All experiments were done in duplicate and the pH was analysed at the end of incubation time.

Substrate utilization spectrum

The ability of strain AK_5 to utilize different carbohydrates was tested using the BM medium supplemented with various substrates. Substrates tested were: xylose, arabinose, glucose, mannose, galactose, fructose, rhamnose, maltose, cellobiose, sucrose, lactose, trehalose, raffinose, starch, cellulose, CMC, avicel, xylan (from oat spelts), glycerol, pyruvate, serine and threonine. All substrates were added from filter sterilized substrates except for xylan, starch, CMC, cellulose and avicel which were autoclaved with the medium. In all cases the concentration of substrates were 20 mM except for xylan, starch, CMC, cellulose and avicel when 2 g L^{-1} were used. Optical density (OD 600 nm) was measured at the beginning and at the end of incubation time (5 days) except for samples containing xylan, starch, CMC, cellulose and avicel where hydrogen production was used as an indicator of positive growth. Where

growth was detected, hydrogen, volatile fatty acids and ethanol were analysed. 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, barley straw (*Hordeum vulgare*) and grass (*Phleum pratense*). Hydrolysates were prepared according to Sveinsdottir et al. (2009), yielding a final dry weight of 22.5 g L^{-1}. Biomass was pretreated chemically by using 0.50 % (v/v) of acid (H_2SO_4) or alkali (NaOH) (control was without chemical pretreatment) before autoclaving (121°C, 60 min). Two commercial enzyme solutions, Celluclast® (750 U/g) and Novozyme 188 (200 U/g) (Sigma), 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. Thereafter, the hydrolysates were incubated in water bath at 45°C for 68h. After the enzyme treatment the pH was measured again and adjusted with NaOH or HCl to pH 6.5 which is the pH optimum of the strain. The hydrolysates were then filtered (Whatman – WeiBrand; 0.45 µm) into sterile bottles.

Fermentation during external electron scavenging systems

In one set of experiments, strain AK_5 was incubated on glucose (20 mM) in the presence of thiosulfate (40 mM) or in co-culture with a hydrogenotrophic methanogen (HT). The HT strain was pre-grown in BM medium with a gas phase consisting of 80% H_2 and 20% CO_2 for one week. Then, the experimental culture bottles were flushed with nitrogen prior to the addition of glucose (20 mM) and inoculation with strain AK_5. The co-culture was incubated at 65°C for four days.

Fermentation of hydrolysates

Fermentation of carbohydrates present in hydrolysates was performed in 24.5 mL serum bottles. The medium (8.0 mL) was supplemented with hydrolysates (2.0 mL –

total liquid volume of 10 mL) giving a final hydrolysate concentration of 4.5 g L^{-1}. Control samples did not contain hydrolysate. In one experiment lower concentrations of hydrolysates were used for fermentation, or 2.25 g L^{-1}.

Analytical methods

Hydrogen, ethanol and volatile fatty acids were measured by gas chromatography as previously described (Orlygsson and Baldursson, 2007). Glucose was determined by slight modification of the method from Laurentin and 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. Absorbance was then measured at 600 nm by using Perkin-Elmer Lambda-25 UV-Vis spectrophotometer.

Results & discussion

The strain AK_5 clearly belongs to the genus *Thermoanaerobacter* (Figure 1). Its closest relative is *T. thermohydrosulfuricus* (99.1% homology) and *T. wiegelii* (98.9%) but it is also closely related to *T. ethanolicus* (98.7%), *T. siderophilus* (98.4%) and *T. acetoethylicus* (98.0%). The genus *Thermoanaerobacter* falls into Clusters V in the phylogenetic interrelationship of *Clostridium* according to Collins et al. (1994). The taxonomy of the genus was refined by Lee et al. (1993) but since then many new species have been described or renamed (e.g. *T. brockii*) (Cayol et al., 1995) or reassigned to a new genus, *Caldanaerobacter* (Fardeau et al. 2004). All species within the genus are obligate anaerobes, ferment various carbohydrates to ethanol, acetate, lactate, hydrogen and carbon dioxide (Lee et al. 1993). Some species can degrade amino acids (Fardeau et al. 1996; Fardeau et al. 1997). Most strains can reduce thiosulfate to hydrogen sulphides which makes these bacteria a potential biocorrosive agents in e.g. the oil petroleum fields (Fardeau et al. 1993). Today, the genus consists of 18 species according to the Euzeby list of prokaryotes.

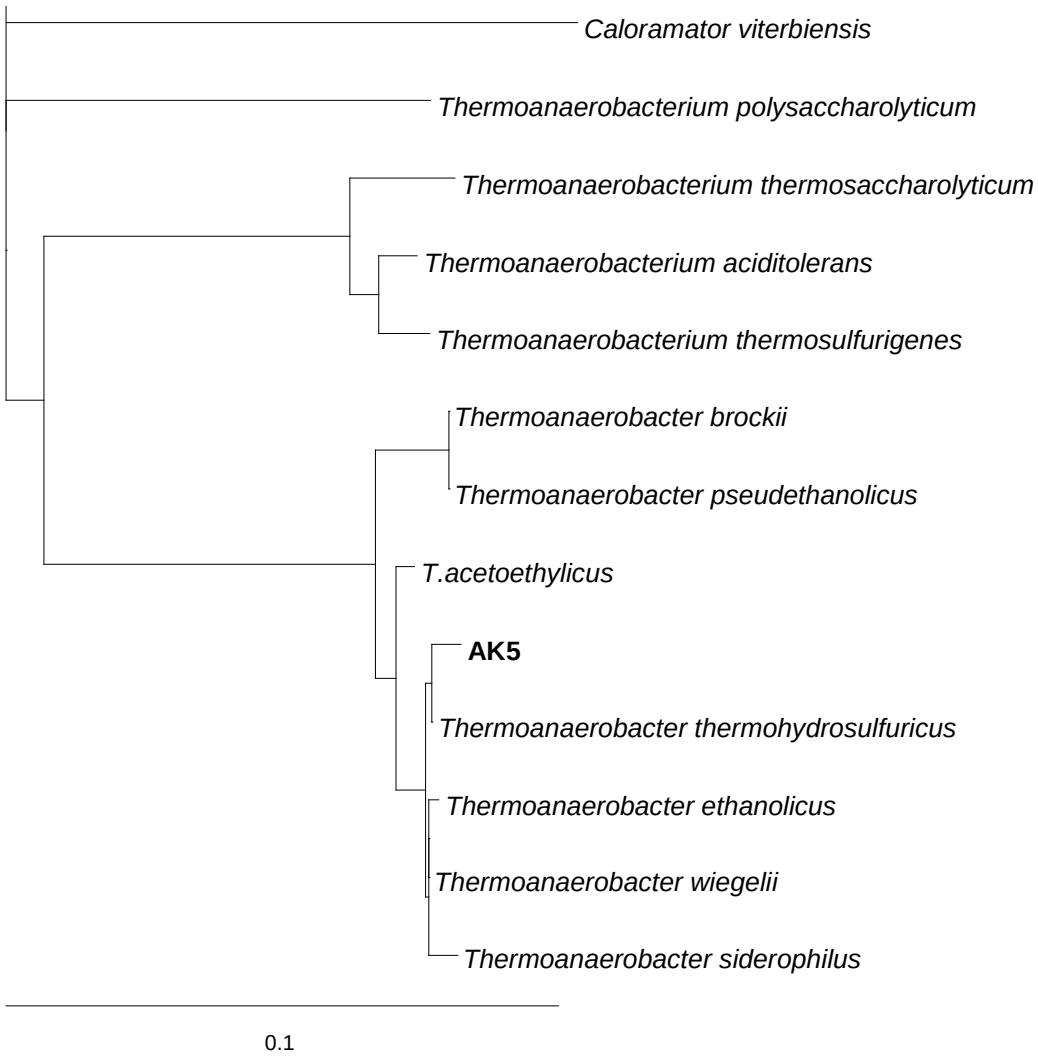

Figure 1. Phylogeny of strain AK5 based on partial 16S rRNA sequence analysis. The phylogenetic tree was generated by using distance matrix and neighbor joining algorithms. Caloramator viterbiensis was selected as outgroup. The bar indicates 0.1 substitutions per nicleotide position.

The hydrogentrophic methanogen (HT) utilized hydrogen at the rate of 0.4 mmol H_2 L^{-1} h^{-1} and cultivation on 40 mmol L^{-1} of hydrogen and 10 mmol L^{-1} carbon dioxide resulted in the formation of 11.0 mmol L^{-1} of methane (during 96h cultivation). The strain was not phylogenetically analysed.

Optimum growth conditions

The strain had a relatively narrow temperature growth range or 60.0°C to 75.0°C with optimal temperature at 65.0°C and a maximum growth rate of 0.362 h. No growth was observed below 60.0°C or above 75.0°C. The pH optimum was 7.0 (maximum growth rate; 0.414 h). Below pH 4.0 and above pH 8.0 no growth was observed.

Kinetic of glucose degradation

The strain has a relatively long lag phase of 26 h with little glucose degradation or end product formation (Figure 2).

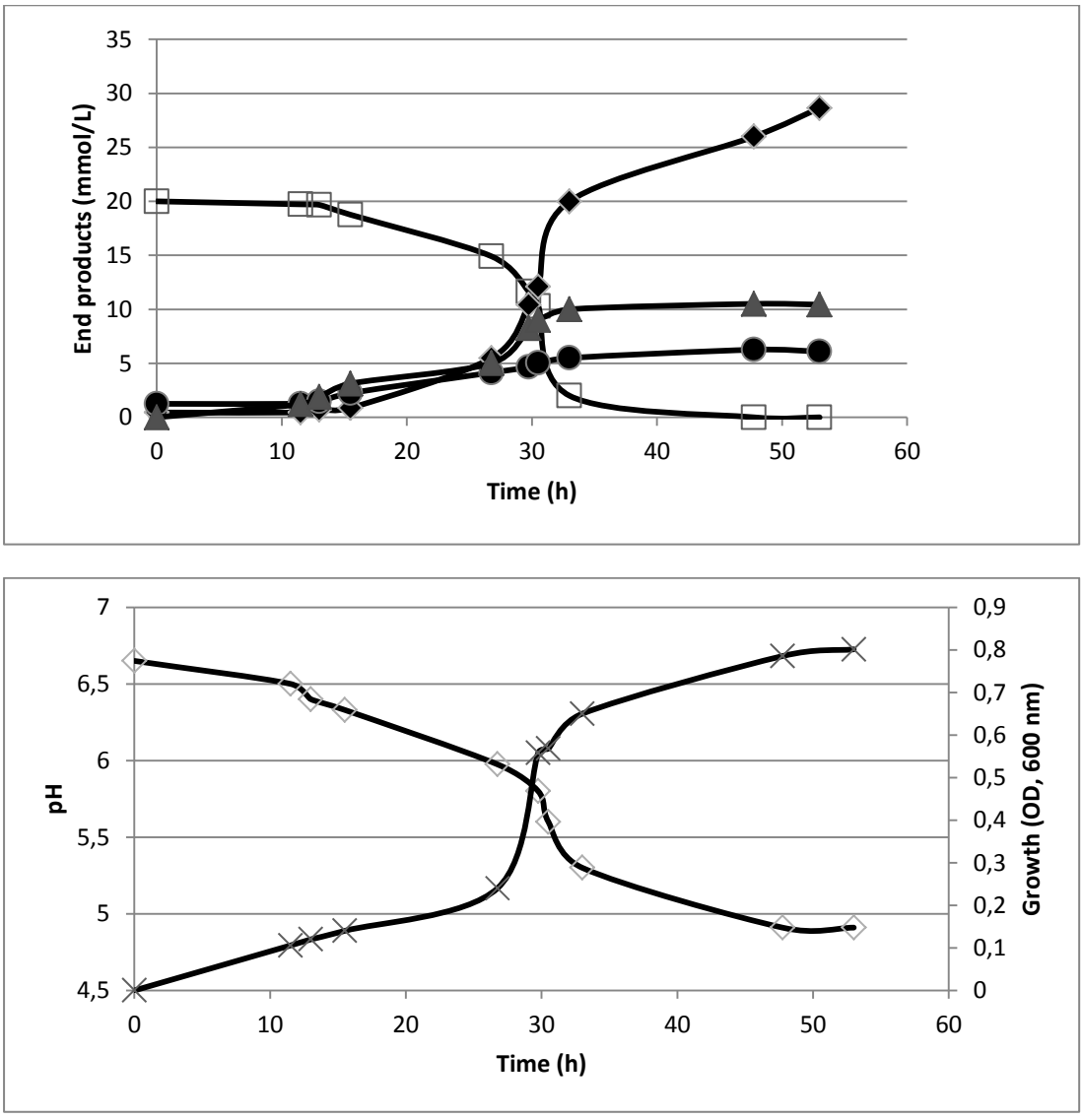

Figure 2. Kinetics of glucose degradation by strain AK5. Glucose (□), Ethanol (♦), Acetate (●), Hydrogen (▲), pH (◊), growth (X).

After 26 h the growth suddenly increases and a dramatic drop in pH occurs and almost all glucose is degraded within 8 h. End product formation from glucose is mainly towards ethanol but also some acetate and hydrogen are produced. Ethanol yields are about 1.4 mol EtOH mol glucose^{-1} degraded.

Ethanol production from sugars and other substrates

One of the main reasons for increased interest in using thermophilic bacteria for second generation ethanol production is because of their broad substrate spectrum. Therefore, it was decided to cultivate the strain on the most common sugars present in lignocellulosic biomass as well as many others. The data from this experiment is presented in Figure 3. Clearly, the strain is a very powerful ethanol producer; it produces 1.60 mol ethanol mol glucose^{-1} and 1.35 mol ethanol mol xylose^{-1} (control values subtracted) or 80.0 and 81.3% of theoretical yields, respectively. These values are similar to other species within the genus and some other bacteria (Fardeau et al., 1996; Sveinsdottir et al., 2009; Wiegel and Ljungdahl, 1981). Slightly higher yields as compare with the kinetic experiment (Figure 2) may be explained by a constant disturbance by removing both gas and volatile samples in that experiment. These high ethanol yields are however not surprising considering that the strain is closely related to *T. ethanolicus* and *T. thermohydrosulfuricus*, e.g. species with excellent ethanol yields from sucrose, glucose and xylose (Table 1). The substrate spectrum of the strain shows a broad capacity in degrading both pentoses (xylose), hexoses (glucose, mannose, galactose, fructose), disaccharides (maltose, cellobiose, lactose, trehalose) as well as starch, pyruvate and serine. In all cases the major end product is ethanol except for serine and pyruvate in which acetate is the primary end product. The highest ethanol concentrations were produced from the disaccharides maltose and lactose (42 - 43 mM).

Table 1. Ethanol production from sugars by pure cultures of Thermoanaerobacter species. Ethanol yields as well as substrate concentrations and incubation temperature are also shown.

Organisms	Sugar	Sugar conc. ($g\ L^{-1}$)	Ethanol Yield (mM mol $sugar^{-1}$)	Temp (°C)	Reference
T. ethanolicus	Glucose	8.0	1.9	72	Wiegel & Ljungdahl (1981)
T. ethanolicus	Glucose	20.0	1.9	68	Carreira et al. (1983)
T. ethanolicus	Xylose	4.0-27.5	0.6-1.3	60	Lacis & Lawford (1988)
T. ethanolicus	Sucrose	15.0-30.0	1.8-3.6	65	Avci et al. (2006)
T. ethanolicus	Xylose	5.0	1.0-1.2	65	He et al. (2010)
T. ethanolicus	Glucose	5.0	1.2-1.3	60	He et al. (2010)
T. thermohydrosulfuricus	Glucose	5.0	1.6	60	Lovitt et al. (1984)
T. thermohydrosulfuricus	Glucose	10.0	1.4-1.9	65	Lovitt et al. (1988)
T. thermohydrosulfuricus	Sucrose	15.0-30.0	1.1.3.0	65	Avci et al. (2006)
Thermoanaerobacter sp. 65-2	Sucrose	15.0-30.0	1.3-3.2	65	Avci et al. (2006)
T. finni	Glucose	Not analysed	1.5	60	Fardeau et al. (1996)
T. finni	Xylose	Not analysed	1.8	60	Fardeau et al. (1996)
Thermoanaerobacter AK33	Glucose	3.6	1.5	70	Sveinsdottir et al. (2009)
Thermoanaerobacter AK33	Xylose	3.6	0.8	70	Sveinsdottir et al. (2009)
Thermoanaerobacter AK5	Glucose	3.6	1.7	65	This study
Thermoanaerobacter AK5	Xylose	3.0	1.3	65	This study

During growth on serine and pyruvate, the carbon flow was shifted away from ethanol to acetate together with hydrogen. This can be explained by the oxidation state of these substrates as compared to e.g. sugars. The oxidation state of the carbon in glucose is zero and during its oxidation to pyruvate the electrons are transferred to NAD^+ leading to the formation of NADH. Reoxidation of NADH to NAD^+ by the strain presumable occurs mainly through acetaldehyde dehydrogenase and alcohol dehydrogenase rendering ethanol as the main product. However, both pyruvate and serine are more oxidized substrates as compared to sugars (glucose) and there is no need to reoxidize NADH. Instead, the strain deaminates serine directly to pyruvate which is decarboxylated to acetyl phosphate (by phosphotransacetylase) and further to acetate (by acetate kinase) resulting in ATP formation. The strain did not degrade arabinose (pentose), rhamnose (hexose), sucrose (disaccharide), raffinose (trisaccharide), xylan, glycerol, threonine or any of the cellulosic substrates tested.

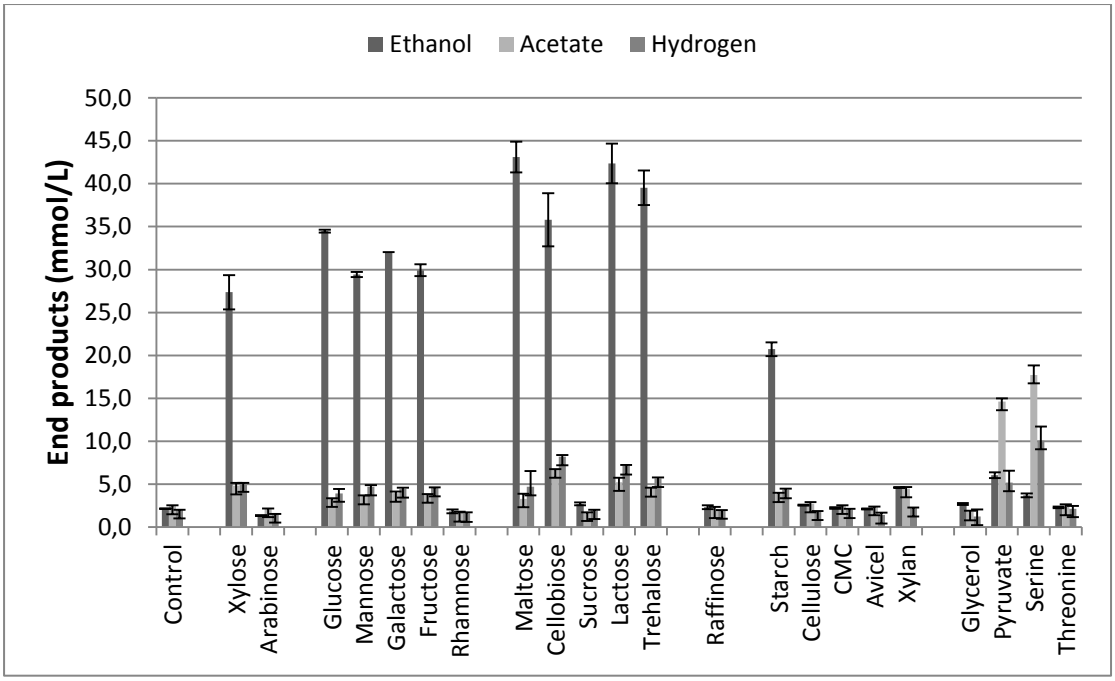

Figure 3. End product formation from various substrates by strain AK5. Data represents average of two replicate experiments. Standard deviations are shown as error bars. From left to right; ethanol, acetate and hydrogen.

Effect of initial glucose loadings on ethanol production

High initial substrate concentration may inhibit substrate utilization and/or decrease end product yields (Lacis and Lawford 1988; Sommer et al., 2004; van Ginkel and Sung 2001). 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 utilization (Olsson and Hahn-Hägerdal, 1996; van Ginkel and Sung, 2001). To investigate the influence of initial substrate concentration on end product formation, changes in pH and substrate degradation, strain AK_5 was cultivated with different concentrations of glucose (0 to 100 mM). The strain degraded all the glucose at lower (5 to 20 mM) initial glucose loadings. At 30 mM loadings, 80% of the glucose was degraded but at 50 mM only 62% glucose degradation occurred and only 20% at 100 mM. This is reflected in decrease in end product formation (Figure 4) and lower pH where the glucose loading is increased.

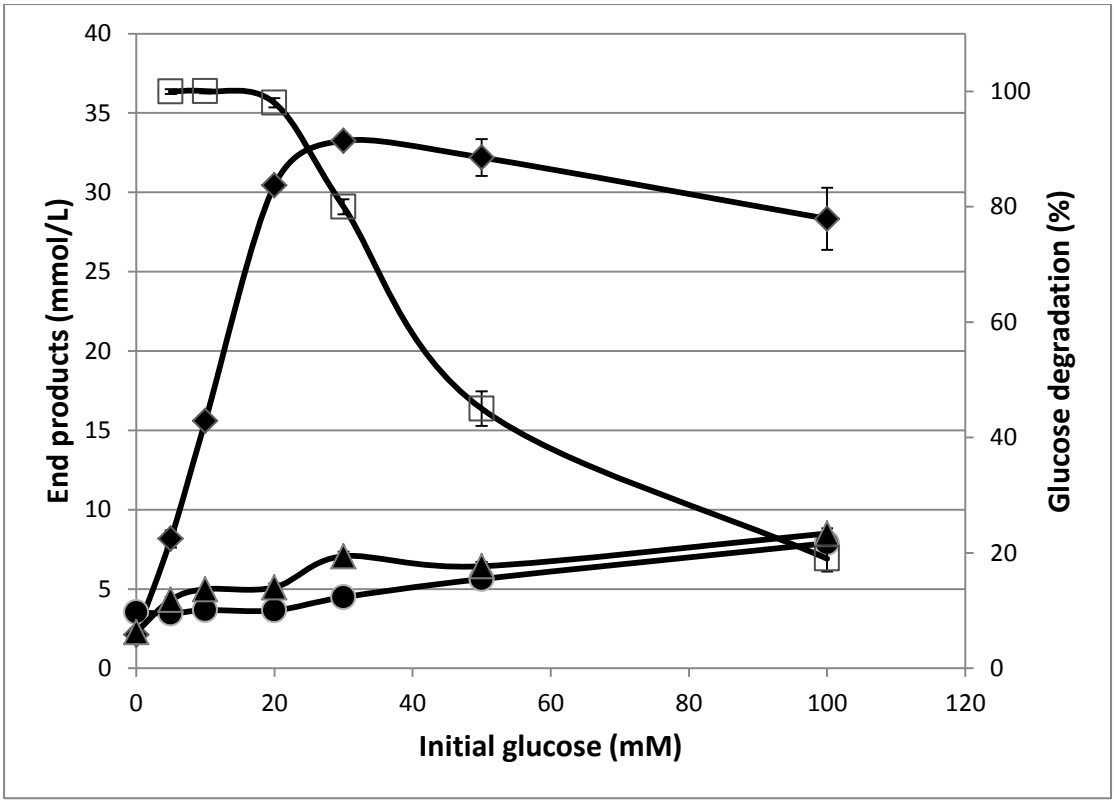

Figure 4. End product formation from different initial glucose concentrations. Also shown are percent of glucose degraded. Values represent means of two replicates and standard deviation are shown as error bars. Glucose (□), Ethanol (♦), Acetate (●), Hydrogen (▲).

The pH was measured after fermentation and went from pH 6.5 at low glucose loadings to pH 4.9 at high (≥ 50mM) loadings. 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.

The effect of partial pressure of hydrogen on end product formation

A change in partial pressure of hydrogen has been reported to affect end product formation of anaerobic bacteria. Thus, at high pH_2, the product formation is directed towards more reduced products like ethanol and lactate but away from acetate and H_2 (Hawkes et al. 2002; Nath & Das 2004; Sigurbjornsdottir and Orlygsson, 2012; van Niel et al. 2006). This was tested in the present study by culturing the strain on glucose (20 mM) with different liquid to gas ratio (range used was from 0.04 to 3.27).

Theoretical yields of hydrogen production is 4 moles of hydrogen per mole of hexose degraded if acetate is the only carbon containing volatile end product (Hawkes et al. 2002). The maximum hydrogen yields for strain AK_5 were much lower, or 1.3 mol H_2 mol glucose^{-1} degraded at the lowest L-G ratio (Figure 5). This is not surprising since the strain is higly ethanolgenic. These yields were however dramatically lowered by increasing the L-G ratios and resulted in a dramatic shift in end product formation, or decrease of acetate and increse of ethanol. Maximum ethanol yields obtained were 1.70 mol ethanol mol^{-1} glucose at the highest L-G ratio used.

By using the fermentation data from the lowest and highest L-G ratios the following equations are observed:

1.0 Glucose → 0.70 EtOH + 0.74 Acetate + 1.30 H_2 + 1.44 CO_2 (low L-G; Eq.1)

1.0 Glucose → 1.70 EtOH + 0.14 Acetate + 0.10 H_2 + 1.62 CO_2 (high L-G; Eq.2)

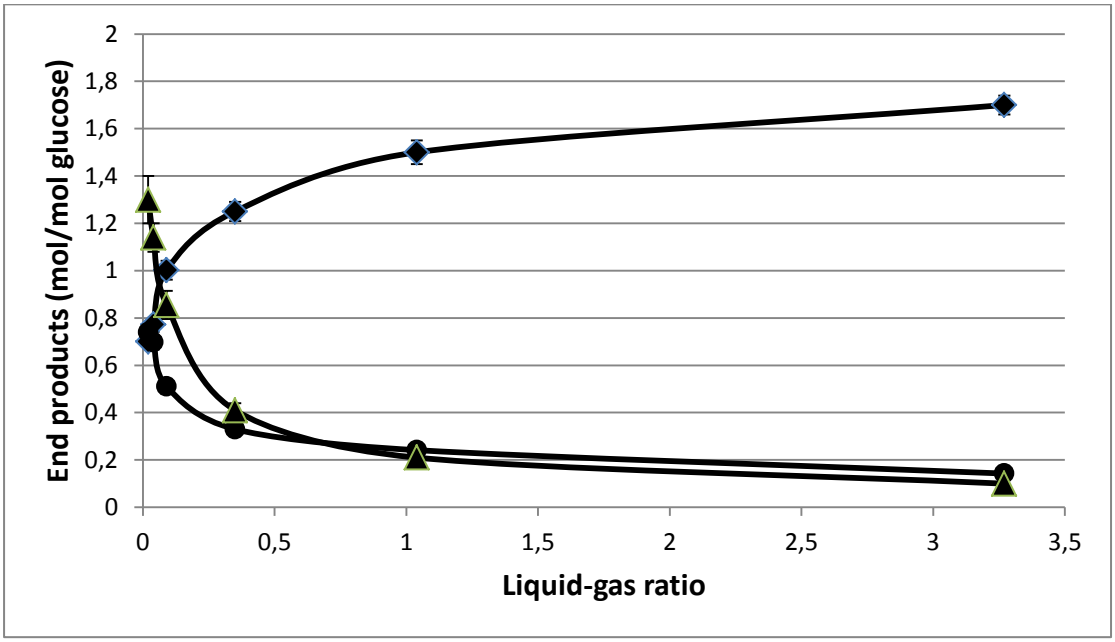

Figure 5. Effect of liquid/gas ration on end product formation from strain AK5. Ethanol (♦), Acetate (●), Hydrogen (▲).Values represent means of two replicates and standard deviations are shown as error bars.

Effect of electron scavenging systems on end product formation.

During the oxidation of glucose to mainly ethanol it is clear from the results above that strain AK_5 disposes of excess electrons via pyruvate through the formation of ethanol. This can be seen from the kinetic of glucose degradation (Figure 2) and from other substrates (Figure 3). However, end product formation is clearly strongly influenced by pH_2 (Figure 5). It is also known that members within the genus *Thermoanaerobacter* can use external electron acceptors like thiosulfate (Fardeau et al. 1993; Fardeau et al. 1996; Fardeau et al. 1997). To investigate further the influence of pH_2 the strain was cultivated under two different electron scavenging systems; a nonbiological system with thiosulfate and a biological system with a co-culture of hydorogenotrophic methanogen. As stated earlier, the strain was producing mainly ethanol from glucose without thiosulfate in pure culture. This was, however, dramatically shifted by using S_2O_3 or a methanogen; in both cases, ethanol became only a minor product and the main volatile end product was acetate (Table 2).

Table 2. Utilization of glucose by strain AK5 in the presence of thiosulfate or a hydrogenotrophic methanogen. Data represents average of two replicate experiments ± standard deviations

	Concentration (mmol L^{-1})			
	Ethanol	Acetate	Hydrogen	Methane
Control	1.7 ± 0.1	2.4 ± 0.1	2.2 ± 0.1	0.0 ± 0.0
Control + S_2O_3	0.9 ± 0.1	3.7 ± 0.2	0.1 ± 0.0	0.0 ± 0.0
Control + Methanogen	0.0 ± 0.5	9.2 ± 0.4	0.0 ± 0.0	3.0 ± 0.0
Glucose	29.1 ± 1.5	10.0 ± 0.3	16.1 ± 0.5	0.0 ± 0.0
Glucose + S_2O_3	5.0 ± 0.3	34.9 ± 2.1	1.9 ± 0.1	0.0 ± 0.0
Glucose + Methanogen	3.4 ± 0.2	34.9 ± 1.2	0.0 ± 0.0	18.0 ± 1.2

Thiosulfate reduction to sulphide is a common characteristic of sulfate reducing bacteria (Le Faou et al. 1990) but it is also a common among thermophilic bacteria that belong to the genera *Thermoanaerobacter* and *Thermoanaerobacterium* (Lee et al. 1993). Fardeau and coworkers (Fardeau et al. 1997) showed a dramatic shift in end product formation by *Thermoanerobacter finni* on glucose when grown on glucose in

the presence and absence of thiosulfate. In that case, both ethanol and lactate decreased during thiosulfate reduction to hydrogen sulphide acetate increased. The influence of using biological hydrogen scavenging systems has also been investigated by *Thermoanaeobacter brockii* during amino acid degradation (Fardeau et al. 1996). Both thiosulfate and the presence of hydrogen scavenging methanogen were crucial for the oxidative deamination of the branched chain amino acids by this strain. However, degradation of more easily degradable amino acid serine was completely degraded in the presence and absence of thiosulfate and *Methanobacterium* sp. although a shift occurred between ethanol and acetate formation.

Fermentation of hydrolysates from lignocellulosic biomass

The strain is producing 26.6 to 27.6 mM of ethanol from 4.5 g L^{-1} of hydrolysates made from cellulose (Table 3). The yields on cellulose (5.5 to 5.7 mM g^{-1} dw) are less as compared to glucose degradation alone. This difference can be explained by incomplete glucose degradation on cellulose hydrolysates but between 8.2 and 10.3 mM of glucose were left in the fermentation broth after fermentation. No sugar was, however, analysed in fermentations with other types of hydrolysates. The main reason for this is likely due to high initial glucose concentrations as observed earlier (Figure 3) on glucose alone. To investigate this further, the strain was incubated in hydrolysates containing less biomass, or 2.25 g L^{-1}. Ethanol yields from cellulose increased to 7.7 mM g^{-1} (dw) and no glucose was detected after fermentation (results not shown). Other end products (acetate and hydrogen) were also produced in the similar proportions as observed on glucose alone. The addition of acid or alkali agents did not increase end product formation yields on cellulose as was clearly observed on most of the lignocellulosic substrates. Highest ethanol yields on the more complex biomass types (without chemical pretreatment) were observed on hemp stem (3.0 mM g^{-1} dw) but lowest on straw (0.9 mM g^{-1} dw). Addition of either acid or alkali increased yields substantially on most of the lignocellulosic biomass tested. The increase was most profound on hydrolysates from straw pretreated with alkali (2.73 times) and acid (2.36 times).

Table 3. Production of end products from hydrolysates (4.5 g L-1) from different biomasses. Values represent mean of two replicates (± standard deviation). na = not analysed

	Concentration (mmol L^{-1})				
Biomass and pretreatment	Ethanol	Acetate	Hydrogen	Glucose (t=0)	Glucose (t=final)
Control	1.9 ± 0.1	2.5 ± 0.1	2.0 ± 0.1	0.0	0.0
Cellulose	27.4 ± 0.6	5.1 ± 0.0	10.1 ± 0.3	26.5 ± 0.1	10.3 ± 0.4
Cellulose - acid	27.6 ± 1.1	4.4 ± 0.4	7.8 ± 0.5	26.9 ± 0.1	9.8 ± 0.5
Cellulose - alkali	26.6 ± 0.9	4.0 ± 0.1	9.0 ± 0.3	27.0 ± 0.1	8.2 ± 0.3
Hemp Stem	15.0 ± 0.2	5.0 ± 0.2	6.3 ± 0.3	na	0.0 ± 0.0
Hemp Stem - acid	16.4 ± 0.9	5.2 ± 1.5	6.6 ± 0.1	na	0.0 ± 0.0
Hemp Stem - alkali	16.8 ± 0.2	5.7 ± 0.1	5.3 ± 0.4	na	0.0 ± 0.0
Hemp Leaf	5.9 ± 0.1	2.8 ± 0.2	2.9 ± 0.1	na	0.0 ± 0.1
Hemp Leaf - acid	7.1 ± 0.1	2.9 ± 0.6	3.3 ± 0.2	na	0.2 ± 0.0
Hemp Leaf - alkali	4.8 ± 0.1	2.9 ± 0.4	2.4 ± 0.2	na	0.0 ± 0.0
Grass	11.6 ± 0.5	4.0 ± 0.1	5.5 ± 0.2	na	0.0 ± 0.0
Grass - acid	21.3 ± 0.7	6.5 ± 0.2	7.4 ± 0.4	na	0.0 ± 0.0
Grass - alkali	14.0 ± 0.5	5.8 ± 0.1	5.4 ± 0.1	na	0.0 ± 0.0
Paper	8.4 ± 0.2	3.2 ± 0.1	4.6 ± 0.1	na	0.0 ± 0.0
Paper- acid	10.4 ± 0.1	3.9 ± 0.4	4.5 ± 0.2	na	0.0 ± 0.0
Paper - alkali	11.8 ± 0.8	4.0 ± 0.2	3.8 ± 0.2	na	0.0 ± 0.0
Straw	5.5 ± 0.3	2.5 ± 0.1	2.9 ± 0.1	na	0.1 ± 0.0
Straw - acid	13.1 ± 0.1	5.1 ± 0.1	6.1 ± 0.2	na	0.1 ± 0.0
Straw - alkali	15.2 ± 1.1	6.9 ± 0.1	6.0 ± 0.1	na	0.0 ± 0.1

The highest ethanol yields by *Thermoanaerobacter* species have been reported by *Thermoanaerobacter* strain BG1L1 on wheat straw (Georgieva et al., 2008) and corn stover (Georgieva et al., 2007), or 8.5 - 9.2 mmol g^{-1} sugar consumed (Table 4). Among other studies with good yields from lignocellulose are e.g. *Clostridium thermocellum* on paddy straw, sorghum stover and corn stubs (Rani et al., 1998) and *Thermoanaerobacter mathranii* on wheat straw (Klinke et al., 2001). Bacteria within the genus *Thermoanareobacterium* have also been shown to have very good ethanol yields on both sugars and lignocellulosic biomass (Almarsdottir et al., 2012). Strain

AK_{17}, produces 8.6 mM g^{-1} cellulose hydrolysate and 5.5 mM g^{-1} grass hydrolysate at very low substrate (2.5 g L^{-1}) concentrations (Almarsdottir et al. 2012). Thus, strain AK_5 has higher yields on glucose (1.7 mol ethanol mol $glucose^{-1}$) as compared to *Thermoanaerobacterium* AK_{17} (1.5 mol ethanol mol $glucose^{-1}$), but yields on cellulose and grass were slightly lower.

*Table 4. Ethanol production from lignocellulosic biomass by pure cultures of Thermoanaerobacter species. Cultivation were either in batch or continuous (con). EtOH yields given in mM/g substrate degraded as well as substrate concentrations and incubation temperature are also shown. * = sugar concentration.*

	Biomass	Substrate conc. (g L^{-1})	Ethanol Yields (mM g $sugar^{-1}$)	Temp. (°C)	Reference
T. ethanolicus	Wood hydrolysate	8.0	3.3 – 4.5	70	Wiegel et al. (1983)
T. mathranii	Wheat straw	6.7	1.3	70	Ahring et al. (1999)
T. mathranii	Wheat straw	6.7	5.3	70	Klinke et al. (2001)
T. etahnolicus	Beet molasses	19.5	4.8	65	Avci et al. (2006)
T. thermohydrosulfuricus 70-1	Beet molasses	19.5	3.0	65	Avci et al. (2006)
Thermoanaerobacter sp. 65-G	Beet molasses	19.5	7.3	70	Avci et al. (2006)
Thermoanaerobacter BG1L1	Corn stover	12.0 – 41.0	8.5-9.2	70	Georgieva et al. (2007)
Thermoanaerobacter BG1L1	Wheat straw	11.0 – 40.0	8.5-9.2	70	Georgieva et al. (2008)
Thermoanaerobacter AK33	Grass	7.5	1.4	70	Sveinsdottir et al. (2009)
Thermoanaerobacter AK33	Cellulose	7.5	3.9	70	Sveinsdottir et al. (2009)
Thermoanaerobacter AK5	Cellulose	2.25	7.7	65	This study

Conclusion

Ethanol production was studied by *Thermoanaerobacter* AK_5 isolated from hot spring in Iceland. The main aim of the study was to investigate the importance of the partial pressure of hydrogen on end product (ethanol) formation by either using high pH_2 or hydrogen scavenging systems. Additionally, ethanol yields from various lignocellulosic biomass hydrolysates were studied. Basic findings of the strain were the following:

- Ethanol yields of 1.70 mol EtOH mol glucose^{-1} and 1.35 mol EtOH mol xylose^{-1}.
- Broad substrate spectra, degrades various hexoses, pentoses, disaccharides and polymeric substrates.
- Inhibited by relatively low initial substrate (glucose) concentrations.
- Is highly flexible for either ethanol or acetate production depending on the culture conditions used.
- Produces up to 7.7 mM EtOH g^{-1} cellulose and 4.4 mM g^{-1} grass hydrolysate.

In summary, the results indicate that the strain is a promising ethanol producer with high yields from lignocellulosic biomass. Ethanol yields can be maximized by cultivating the strain under high pH_2 hydrogen. The main drawback of the strain is its inhibition of substrate (glucose) utilization at elevated substrate concentrations. This limitation could be solved by cultivating the strain in fed batch or continuous mode.

Acknowledgements

This work was sponsored by RANNÍS, (Technology Development Fund, projects 081303408 (BioEthanol) and RAN091016-2376 (BioFuel)), and The Research Fund of the University of Akureyri.

References

Ahring BK, Licht D, Schmidt AS, Sommer P. 1999. Production of ethanol from wet oxidised wheat straw by *Thermoanaerobacter mathranii*. Bioresource Technol 68:3-9

Almarsdottir AR, Taraceviz A, Gunnarsson I, Orlygsson J. 2010. Hydrogen production from sugars and complex biomass by *Clostridium* species, AK14, isolated from Icelandic hot spring. Icelandic Agric Sci.23:61-71.

Almarsdottir AR, Sigurbjornsdottir MA, Orlygsson J. 2012. Effect of various factors on ethanol yields from lignocellulosic biomass by Thermoanaerobacterium AK17. Biotech Bioeng 109:686-694.

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

Carreira LH, Wiegel J, Ljungdahl LG. 1983. Production of ethanol from bio-polymers by anaerobic, thermophilic, and extreme thermophilic bacteria. I. Regulation of carbohydrate utilization in mutants of *Thermoanaerobacter ethanolicus*. Biotechnol Bioeng Symph.13:183-191.

Carlier J-P, Bonne I, Bedora-Faure M. 2006. Isolation from canned foods of *Thermoanaerobacter* species phylogenetically related to *Thermoanaerobacter mathranii* (Larsen 1997): Emendation of the species description and proposal of *Thermoanaerobacter* subsp. *Alimentarius* subsp. nov. Anaerobe 12:153-159.

Cayol J-L, Ollivier B, Patel BKC, Ravot G, Magot M, Ageron E, Grimont PAD, Garcia J-L. 1995. Description of *Thermoanerobacter brockii* subsp. *lactoethylicus* supsp. nov., isolated from a deep surface French oil well, a proposal to reclassify *Thermoanerobacter finnii* as *Thermoanaerobacter brockii* subsp. *finnii* comb. *Thermoanaerobacter brockii*. Int J Syst Bacteriol 45:783-789.

Collins MD, Lawson PA, Willems A, Cordoba JJ, Garayzabal FJ, Garcia P, Cai, J, Hippe H, Farroe JAE. 1994. The phylogeny of the genus *Clostridium* – Proposal of 5 new genera and 11 new species combinations. Int J Syst Microbiol 44:812-826.

Fardeau M-L, Cayol J-L, Magot M, Ollivier B. 1993. Hydrogen oxidation abilities in the presence of thiosulfate as electron acceptor within the genus *Thermoanaerobacter*. Current Microbiol 30:1-6.

Fardeau M-L, Faudon C, Cayol J-L, Magot M, Patel B.K.C, Ollivier B. 1996. Effect of thiosulfate as electron acceptor on glucose and xylose oxidation by *Thermoanerobacter finnii* and a *Thermoanaerobacter* sp. isolated from oil field. Water Rersearch Microbiol 147:159-165.

Fardeau M-L, Patel B.K.C, Magot M, Ollivier B. 1997. Utilization of serine, leucine, isoleucine, and valine by *Thermoanaerobacter brockii* in the presence of thiosulfate or *Methanobacterium* sp. as electron acceptors. Anaerobe 3:405-410.

Fardeau M-L, Bonilla Salinas M, L'Haridon S, Jeanthon V, Verhé F, Cayol JL, Patel BKC, Garcia JL, Ollivier B. 2004. Isolation from oil reservoirs of novel thermophilic anaerobes phyogenetically related to *Thermoanaerobacter subterraneus*: reassignment of *T. subterraneus*, *Thermoanaerobacter*

yonseiensis, *Thermoanaerobacter tengcongensis* and *Carboxydibrachimum pacifium* to *Caldanaerobacter subterraneous* gen.nov., sp.nov., comb. Nov. as four novel subsepcies. Int J Syst Evol Microbiol 54:467-474.

Georgieva TI, Ahring BK. 2007. Evaluation of continuous ethanol fermentation of dilute-acid corn stover hydrolysate using thermophilic anaerobic bacterium *Thermoanaerobacter* BG1L1. Appl Microbiol Biotechnol 77:61-68.

Georgieva TI, Mikkelsen MJ, Ahring BK. 2008. Ethanol production from wet-exploded wheat straw hydrolysates by thermophilic anaerobic bacterium *Thermoanaerobacter* BG1L1 in a continuous immobilized reactor. Appl Biochem Biotechnol 145:99-110.

Gírio FM, Fonseca C, Carvalheiro F, Duarte LC, Marques S, Bogel-Lukasik R. 2010. Hemicelluloses for fuel ethanol: A review. Bioresource Technol 101:4475-4800.

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

Hawkes FR, Dinsdale R, Hawkes DL, Hussy I. 2002. Sustainable fermentative hydrogen production: challenges for process optimisation. Int J Hydrogen Energy 27:1339-1347.

He Q, Lokken PM, Chen S, Zhou J. 2009. Characterization of the impact of acetate and lactate on ethanolic fermentation by *Thermoanaerobacter ethanolicus*. Bioresource Technol 23:5955-5965.

Koskinen PEP, Beck SR, Orlygsson J, Puhakka JA. 2008. Ethanol and hydrogen production by two thermophilic, anaerobic bacteria isolated from Icelandic geothermal areas. Biotechnol Bioeng 101:679-690.

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

Lacis LS, Lawford HG. 1988. Ethanol production from xylose by *Thermoanaerobacter ethanolicus* in batch and continuous culture. Arch Microbiol 150:48-55.

Laurentin A, Edwards CA. 2003. A microtiter modification of the anthrone-sulfuric acid colorimetric assay for glucose-based carbohydrates. Anal Biochem 315:143-145.

Lee YE, Jain MK, Lee C, Lowe SE, Zeikus JG. 1993. Taxonomic distinction of saccharolytic thermophilic anaerobes: Description of *Thermoanaerobacterium xylanolyticum* gen. nov., sp. nov., and *Thermoanaerobacterium saccharolyticum* gen. nov., sp. nov.; reclassification of *Thermoanaerobium brockii*, *Clostridium thermosulfurogenes*, and *Clostridium thermohydrosulfuricum* E100-69 as *Thermoanaerobacter brockii* comb. nov., *Thermoanaerobacterium thermosulfurigenes* comb. nov., and *Thermoanaerobacter thermohydrosulfuricus* comb. nov., respectively; and transfer of *Clostridium thermohydrosulfuricum* 39E to *Thermoanaerobacter ethanolicus*. Int J Syst Bacteriol 43:41-51.

Le Faou A, Rajagopal BS, Daniels L, Fauque G. 1990. Thiosulphate, polythionates and elemental suophur assimilation and reduction in the bacterial world. FEMS Microbiol Rev 75:351-382.

Lovitt R.W, Longin R, Zeikus JG. 1984. Ethanol production by thermophilic bacteria: physiological comparison of solvent effects on parent and alcohol-tolerant strains of *Clostridium thermohydrosulfuricum*. Appl Environ Microbiol 48:171-177.

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

Nath K, Das D. 2004. Improvement of fermentative hydrogen production: Various approaches. Appl Microbiol Biotechnol 65:520-529.

Olsson L, Hahn-Hagerdahl B. 1996. Fermentation of lignocellulosic hydrolysates for ethanol production. Enzyme Microb Tech 18:312-331.

Orlygsson J, Baldursson SRB. 2007. Phylogenetic and physiological studies of four hydrogen-producing thermoanaerobes from Icelandic geothermal areas. Icelandic Agric Sci 20:93-106.

Orlygsson J, Sigurbjornsdottir MA, Bakken HE. 2010. Bioprospecting thermophilic ethanol and hydrogen producing bacteria from hot springs in Iceland. Icelandic Agric Sci 23:73-85.

Rani SK, Swamy MV, Seenayya G. 1998. Production of ethanol from various pure and natural cellulosic biomass by *Clostridium thermocellum* strains SS21 and SS22. Process Biochem 33:435-440.

Sommer P, Georgieva T, Ahring BK. 2004. Potential for using thermophilic anaerobic bacteria for bioethanol production from hemicellulose. Biochem Soc Trans 32:283-289.

Sigurbjornsdottir MA, Orlygsson J. 2012. Combined hydrogen and ethanol production from sugars and lignocellulosic biomass by *Thermoanaerobacterium* AK54. Applied Energy. Doi:10/1016/J.apenergy.2011.11.035.

Sveinsdottir M, Baldursson SRB, Orlygsson J. 2009. Ethanol production from monosugars and lignocellulosic biomass by thermophilic bacteria isolated from Icelandic hot springs. Icelandic Agric Sci 22:45-58.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876-4882.

van Ginkel S, Sung S. 2001. Biohydrogen production as a function of pH and substrate concentration. Environ Sci Technol 35:4726-4730.

van Groenestijn JW, Hazewinkel JH, Nienoord M, Bussmann PJ. 2002. Energy aspects of biological hydrogen production in high rate bioreactors operated in the thermophilic temperature range. Int. J Hydrogen Energy 27:1141-1147.

van Niel EWJ, Claassen PAM, Stam AJM. 2006. Substrate and end product inhibiton of hydrogen production by the extreme thermophile, *Caldicellulosiruptor saccharolyticus*. Biotechnol Bioeng 121:515-533.

Wiegel J. 1980. Formation of ethanol by bacteria. A pledge for use of extreme thermophilic anaerobic bacteria in industrial ethanol fermentation processes. Experientia 36:1434-1446.

Wiegel J, Ljungdahl LG. 1981. *Thermoanaerobacter ethanolicus* gen. nov., spec. nov., a new, extreme thermophilic, anaerobic bacterium. Arch. Microbiol 128:343-348.

Wiegel J, Carreira LH, Mothershed CP, Puls J. 1983. Production of ethanol from bio-polymers by anaerobic, thermophilic, and extreme thermophilic bacteria. II. *Thermoanaerobacter ethanolicus* JW200 and its mutants in batch cultures and resting cell experiments. Biotechnol Bioeng 13:193-205.

5 Manuscript II

Hydrogen production from sugars and complex biomass by *Thermoanaerobacter* CMC_{15}

Hydrogen production from sugars and complex biomass by *Thermoanaerobacter* CMC_{15}

Hronn BRYNJARSDOTTIR, Johann ORLYGSSON,

University of Akureyri, Faculty of Natural Resource Sciences

Mailing address: Borgir, Nordurslod, 600 Akureyri, Iceland

Address: Faculty of Natural Resource Science, University of Akureyri, Borgir, Norduslod, 600 Akureyri, Iceland. Tel: +354 4608511. Fax: + 354 4609889.

E-mail address: jorlygs@unak.is

Abstract

The hydrogen production capacity of the thermophilic strain CMC_{15} was studied in batch culture. According to partial 16S rRNA gene sequence analysis the strain belongs to the genus *Thermoanaerobacter* and is most closely related to *T. yonseiensis* (98.9% homology) and *T. keratinophilus* (98.5%). The growth of strain HLG_{15} was observed at temperatures between 50 and 75°C with optimal growth between 65 and 70°C. The optimum pH for growth was between pH 6 to 7 but growth was observed from pH 4 to 8. The strain converts various carbohydrates to acetate, ethanol, hydrogen and carbon dioxide. The effect of substrate (glucose) inhibition was investigated and good correlation was observed between increased substrate loadings and end product formation up to 10 mM; at glucose concentrations ≥ 10 mM glucose the substrate was not completely degraded. Also, the pH at the end of fermentation dropped from 6.5 (control without glucose) to 5.5 (at ≥ 50 mM glucose). By varying the liquid to gas phase during glucose fermentation in a batch a clear correlation was found between increased acetate and hydrogen production at low gas-to-liquid conditions and less ethanol. End product formation from hydrolysates made from lignocellulosic (4.5 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 acid (HCl) or a base (NaOH) as well as enzymes (Celluclast® and Novozyme 188). The strain produced the most hydrogen (5.8 to 6.0 mol-H_2 g^{-1}) from cellulose hydrolysates but less from newspaper and lignocellulosic biomasses (2.52 to 4.83 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 7.6 mol H_2 g^{-1} dw. Other end products were acetate, ethanol and carbon dioxide.

Keywords: Hydrogen, *Thermoanaerobacter*, carbohydrates, lignocellulose, hydrolysates

Correspondence to: Orlygsson J

Introduction

Currently, most of the world's energy demand is met by nonrenewable fossil fuels. Energy consumption is growing at an increasing rate leading to increased interest of renewable alternatives to fossil-based fuels (Saxena et al. 2009; Zaldivar et al. 2001; Sánchez & Cardona 2008). Biohydrogen has a great potential as a clean, renewable energy carrier with 2.75 times higher energy density compared to hydrocarbon fuels and produces water as the sole end product after combustion (Levin et al. 2004; Kapdan & Kargi 2006; van Groenestijn et al. 2002). Most of the hydrogen produced today is derived from natural gases via the steam reformation of methane and other hydrocarbons (Turner, 2004).

Production of biohydrogen (and bioethanol) through microbial fermentation are well known processes. Both mesophilic bacteria within the genera of strict anaerobes e.g. *Clostridium* (Taguchi et al. 1992; Kataoka et al. 1997; Almarsdottir et al. 2010) and factultative anaerobes e.g. *Citrobacter* and *Enterobacter* (Yokoi et al. 1995; Oh et al. 2003) have been extensively studied for this purpose. The hydrogen production yields of these bacteria are often in the range of 1 to 2 mol H_2 mol hexose degraded or far away from the theoretical maxium of 4 mol H_2 mol glucose degraded (Nandi & Sengupta, 1998). The main reason for this is because of thermodynamic hindrance at lower temperatures due to the fact that H_2 production from either ferredoxin or NAD(P)H are thermodynamically unfavourable (Jones, 2008; Hallenbeck, 2009). Therefore, at low temperatures, elevated H_2 concentrations inhibit H_2 formation at much lower concentrations than at higher temperatures. Mesophilic and moderate thermophilic bacteria respond to this by directing their reducing equivalents to other more favourable electron acceptors and consequently produce reduced products like ethanol, lactate, butyrate and alanine (van Niel et al. 2003). At high temperatures, the influence of the partial pressure of H_2 is less on the key enzymes responsible for H_2 production leading to fewer end products and higher hydrogen yields (van Groenestijn, 2002; van Niel et al. 2003).

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

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

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

High values of hydrogen produced per mol of glucose utilized have been reported for the hyperthermophiles *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii* or 3.3 to 4.0 mol-H_2 mol carbohydrate (van Niel . et al. 2002; Schroder et al. 1994; Zeidan & van Niel 2010).In an enrichment culture isolated from Icelandic hot spring Koskinen *et al* got up to 3.2 mol-H_2 mol-glucose-1 in a semi-continous batch reactor and 2.10 mol-H_2 mol-glucose^{-1} in batch culture (2008a; 2008b).

Most of the above mentioned studies used simple sugars but not complex biomass or agricultural residues. Lignocellulose is composed of cellulose, hemicellulose and lignin and constitutes about 50% of Earth's biomass (Renewable Fuels Association, 2011). It is the largest source of renewable carbon on Earth and it is not used as a food source for people and is therefore a potential candidate as a substrate for biofuel production (Balat, 2010). One of the main reasons for increased interest in the use of thermophiles, apart from thermodynamics and fewer end products formed, is the fact that they have a broader substrate spectrum as compared to most mesophilic bacteria and may therefore be good candidates for hydrogen production from ligncoellulosic biomass (Almarsdottir et al 2012; Sigurbjornsdottir & Orlygsson, 2012). The lignin fraction of the lignocellulose needs to be removed and the hemicellulose and cellulose fraction hydrolysed, before fermentation. The main sugars after chemical and enzymatic pretreatment are various hexoses and pentoses that are in turn degraded to various end products depending on the culture conditions and microbes used.

Hot springs are a potential source for hydrogen producing microorganisms. In this study, a thermophilic bacterium, isolated from an Icelandic hot spring, with hydrogen and acetate as its main end products, was studied. The fermentation of

various carbohydrates and hydrolysates from several types of lignocellulosic biomasses was investigated. The optimal conditions for hydrogen production in terms of the partial pressure of hydrogen (pH_2), temperature and pH were explored and kinetic parameters for glucose degradation determined.

Materials and methods

Medium

The medium (per liter) consisted of: NH_4Cl 0.3 g, NaCl 0.3 g, $CaCl_2$ 0.11 g, $MgCl_2$ x $6H_2O$ 0.1 g, yeast extract 2.0 g, resazurin 1 mg, trace element solution 1 ml, vitamin solution 1 ml and $NaHCO_3$ 0.8 g. Phosphate buffers were also used where 1 M stock solutions of NaH_2PO_4 and Na_2HPO_4 were made and added to the media to give a buffer capacity of 30 mM. The vitamin solution was according to DSM141. The trace element solution was as described earlier by Örlygsson and Baldursson (2007). The medium was prepared by adding the phosphate buffer, yeast extract and resazurin to distilled water, which was then boiled for 5-10 min and cooled while flushing with nitrogen. The mixture was then transferred to cultivation bottles and autoclaved for 60 minutes. All other components of the medium were added separately through filter-sterilized solutions. All experiments were performed at 65°C at pH 7.0 without agitation. The inoculum volume was 2% in all experiments. All experiments were done in duplicates. The medium hereafter is referred as BM medium.

Isolation of strain

The strain was isolated from a hot spring (70.1°C, pH 7.0) in Grensdalur, S-W Iceland. Samples were collected by using an extended pole with grip arms placed at the end. Sterile serum bottles (117.5 mL) were fixed at the end, opened and completely filled with geothermal mud/liquid sample and closed with butyl rubber and aluminium caps and transferred to the laboratory. Pure cultures were obtained by repeated enrichments and end point dilutions (Orlygsson and Baldursson, 2007) on hydrolysate (HL) made from grass (*Phleum pratense*). The concentration of the HL

was 17.5 g L^{-1} (dw). The HL was made by using enzymatic hydrolysis but without chemical pretreatment (see later).

Optimum growth conditions and kinetics of glucose degradation

To determine the strain's optimum pH for growth the initial pH was set to various levels in the range of 3.0 to 9.0 with increments of 1.0 pH unit. The experimental bottles were supplemented with acid (HCl) and alkali (NaOH) to set the pH accordingly. To determine the optimum temperature for growth the incubation temperature varied from 35°C to 80°C. For the pH optimum the strain was cultivated at 65°C and for the temperature optimum the pH was 7.0. Optimal pH and temperature were used in all experiments performed. Experiments were done in 117.5 mL serum bottles with 50 mL liquid medium. In one set of experiment, kinetics of the strain was done on glucose (20 mM) were gaseous (0.2 mL) and liquid (1.0 mL) samples were withdrawn at certain time intervals and glucose, end products, growth (optical density) as well as pH were analysed.

Phylogenetic analysis

Partial 16S rRNA analysis of 716 nucleotide long sequence was done according to Orlygsson and Baldurson (2007) and references therein. Sequences from 16S rRNA analysis were compared to sequences in the NCBI database using the nucleotide-nucleotide BLAST (BLASTN) tool. The most similar sequences were aligned with the sequencing results in the programs BioEdit (Hall, 1999) and CLUSTAL_X (Thompson et al., 1997). Finally, the trees were displayed with the program TreeView. *Caloramator viterbiensis* was used as an outgroup.

Effect of initial glucose concentration on end product formation

The effect of glucose concentration on strain HLG_{15}, 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 60 mL liquid medium. All experiments were done in duplicates and the pH was analysed at the end of incubation.

Substrate utilization spectrum

The ability of strain CMC_{15} to utilize different carbohydrates was tested using the BM medium supplemented with various substrates in 24.5 mL serum bottles with 10 mL liquid medium. Substrates tested were: xylose, arabinose, glucose, mannose, galactose, fructose, rhamnose, maltose, cellobiose, sucrose, lactose, trehalose, raffinose, starch (from potato), cellulose, carboxymethyl cellulose (CMC), avicel, xylan (from oat spelts), glycerol, pyruvate, serine and threonine.. All substrates were added from filter-sterilized substrates except for xylan, starch, CMC, cellulose and avicel which were autoclaved with the medium. In all cases, the concentration of substrates was 20 mM except for xylan, starch, CMC, cellulose and avicel where 2 g L^{-1}. Optical density (OD 600 nm) was measured at the beginning and at the end of incubation time (5 days) except for samples containing xylan, starch, CMC, cellulose and avicel where hydrogen production was used as an indicator of positive growth. Where growth was detected, hydrogen, volatile fatty acids and ethanol were analysed.

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*). HLs were prepared according to Sveinsdottir et al. (2009), yielding a final dry weight of 22.5 g L^{-1}. Biomass was pretreated chemically by using 0.50 % of acid (H_2SO_4) or alkali (NaOH) (control was without chemical pretreatment) before autoclaving (121°C, 60 min). Two commercial enzyme solutions, Celluclast® (750 U/g) and Novozyme 188 (200 U/g) (Sigma), 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. Thereafter, the hydrolysates were incubated in water bath at 45°C for 68h. After the enzyme treatment the pH was measured again

and adjusted to pH 7.0 which is within 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 (8.0 mL) was supplemented with hydrolysates (2.0 mL – total liquid volume of 10 mL) giving a final hydrolysate concentration of 4.5 g L^{-1}. Control samples did not contain hydrolysate.

Analytical methods

Hydrogen, ethanol and volatile fatty acids were measured by gas chromatography as previously described (Orlygsson and Baldursson, 2007). Glucose was determined by slight modification of the method from Laurentin and Edwards (2003); liquid sample (400 µL) was mixed with 2 mL of anthrone solution (0.2% w/v in 72% sulphuric acid). The sample was boiled for 11 minutes and then cooled down on ice. Absorbance was measured at 600 nm by using Perkin-Elmer Lambda-25 UV-Vis spectrophotometer.

Results and discussion

Phylogeny

The 16S rRNA analysis of strain CMC_{15} showed that it belongs to the genus *Thermoanaerobacter* (Figure 1). Its closest relatives are *T. yonseiensis* (99.9% homology), *T. keratinophilus* (99.5%), *T. subterraneus* (98.2%) and *T. tengcongensis* (98.0%). The genus *Thermoanaerobacter* falls into Clusters V in the phylogenetic interrelationship of *Clostridium* according to Collins et al. (1994). The taxonomy of the genus was refined by Lee et al. (1993) but since then many new species have been described or renamed (e.g. *T. brockii*) (Cayol et al., 1995) or reassigned to a new genus, *Caldanaerobacter* (Fardeau et al. 2004). All species within the genus are

obligate anaerobes, ferment various carbohydrates to ethanol, acetate, lactate, hydrogen and carbon dioxide (Lee et al. 1993). Some species can degrade amino acids (Fardeau et al. 1997). Most strains can reduce thiosulfate to hydrogen sulphides whereas *Thermoanerobacterium* produces sulphur from thiosulfate reduction (Lee et al. 1993). Today, the genus consists of 18 species according to the Euzeby list of prokaryotes.

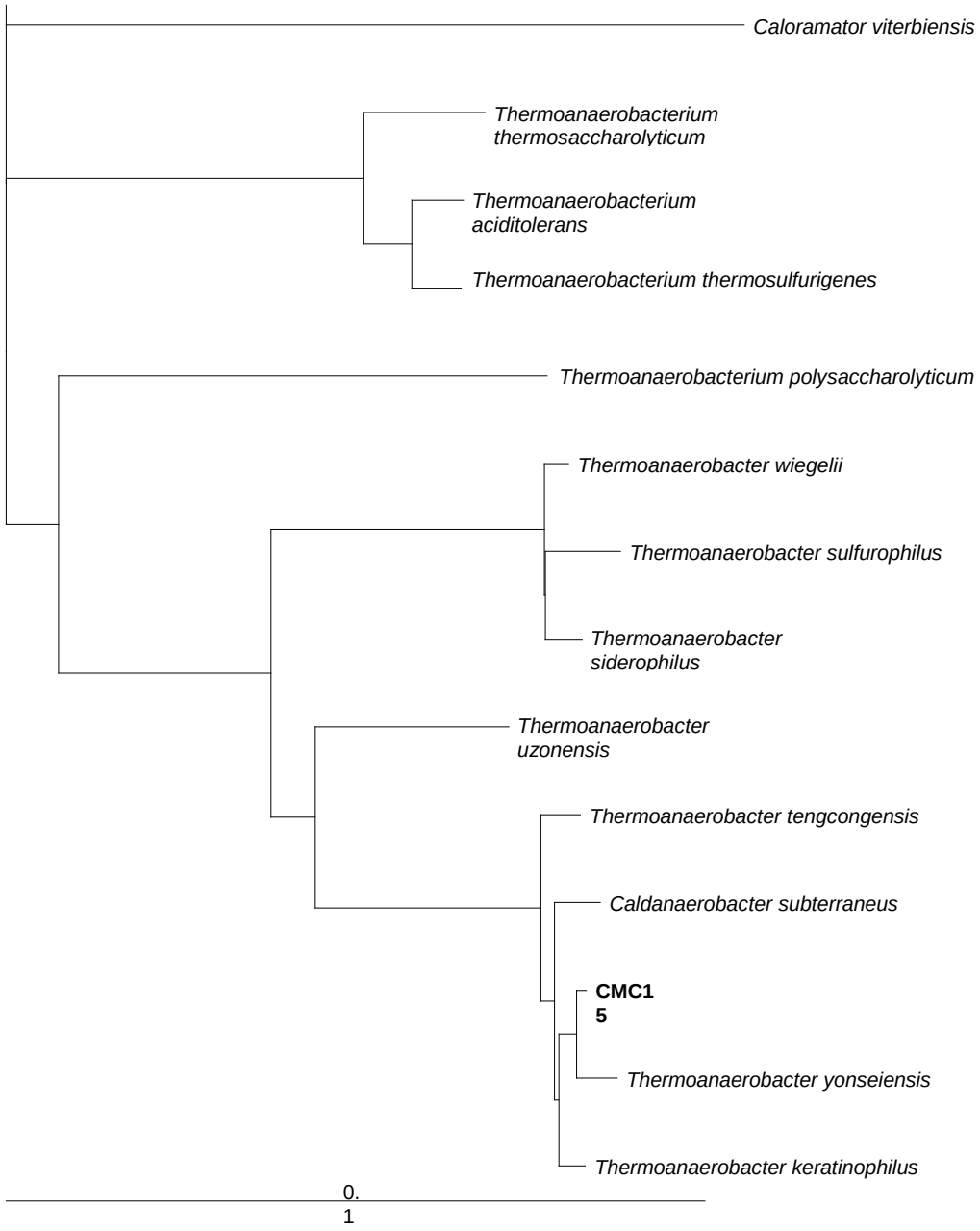

Figure 1. Phylogeny of strain CMC_{15} based on the 16S rRNA gene sequence (716 bp). The phylogenetic tree was generated using a distance matrix and neighbour joining algorithms with 500 bootstraps. Caloramator viterbiensis was used as out-group. The scale bar indicates 0.1 substituitions per nucleotide position.

Optimum growth conditions

The strain grows between 55.0°C to 75.0°C with optimal temperature at 65.0°C with maximum growth rate of 0.076 h. No growth was observed below 55.0°C or above 75.0°C. The pH optimum was between 6.0 and 7.0 with maximum growth rate at 0.096 h). Below pH 3.0 and above pH 9.0 no growth was observed.

Kinetic of glucose degradation

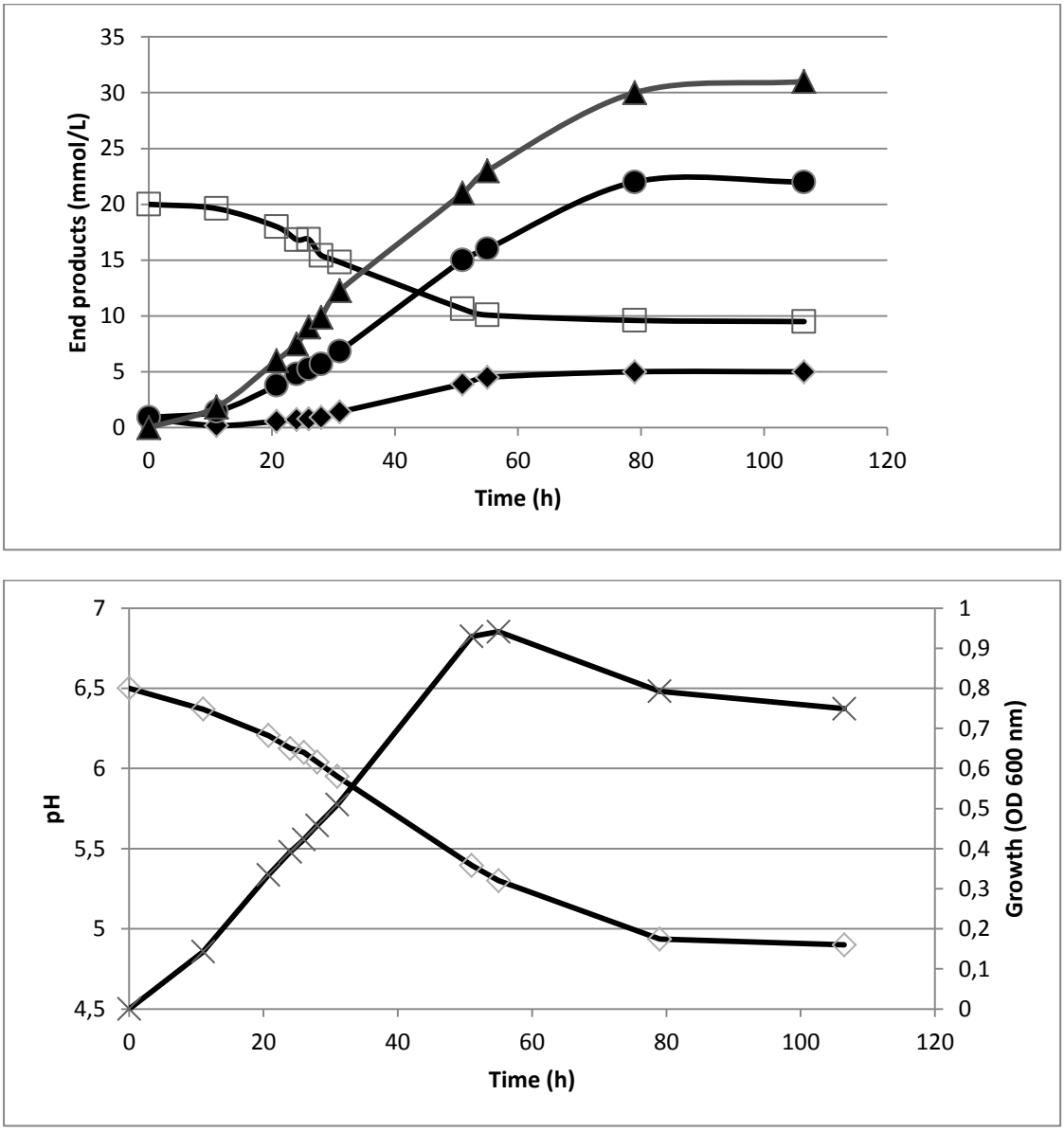

Figure 2. Kinetics of glucose degradation by strain CMC_{15}. Glucose (□), Ethanol (♦), Acetate (●), Hydrogen (▲), pH (◊), growth (X).

The calculated doubling time of the strain is 9.6 h and hydrogen production rate of 0.6 mmol L^{-1} h^{-1} (Figure 2). During growth, the optical density increased to 0.95 but only 10 mM of the glucose was degraded. The only end products analysed in the fermentation broth were ethanol, acetate and hydrogen. The high amounts of both acetate and hydrogen may be explained by relatively high background values produced from yeast extract by the strain and are included in the figure. Thus, by subtracting both the values attributed to yeast extract (acetate 6.5 mM and hydrogen 5.5 mmol L^{-1}) the following stoichiometry was observed:

1.0 Glucose → 1.33 Acetate + 0.15 EtOH + 2.10 H_2 + 1.48 CO_2

The relatively slow growth seems to be linear instead of exponential, most likely due to accumulation of acetate and the rapid decrease in pH in the medium. The pH at the start of the experiments is 6.5 but drops gradually to 4.9. The carbon balance is 83.3% but approximately 11% are likely in the biomass. Repeated sampling (volatiles and gases) may also contribute to low carbon balances.

Hydrogen production from sugars and other substrates

One of the main reasons for increased interest in using thermophilic bacteria for production of second generation biofuels is their broad substrate spectra (Taylor et al. 2008; Almarsdottir et al.,2010; Sigurbjornsdottir & Orlygsson, 2012). To investigate this ability for strain CMC_{15} various carbon substrates were tested for both degradation and end product formation (Figure 3). The strain is indeed quite versatile and degrades most of the carbohydrates tested to the same end products as for glucose, i.e. acetate, ethanol and hydrogen (together with carbon dioxide which was not measured).

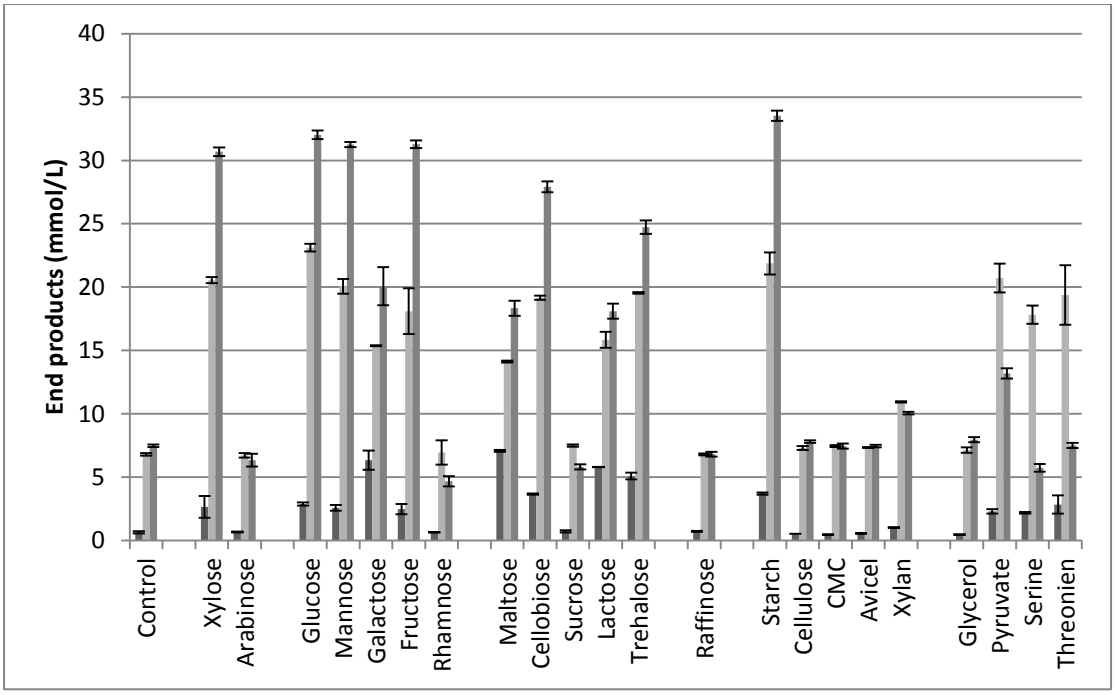

Figure 3. End product formation from various substrates by strain HLG_{15}. Data represents average of two replicate experiments. Standard deviations are shown as error bars. From left to right; ethanol, acetate and hydrogen.

As for glucose, the ethanol productions from other sugars were usually 5 mM or less. Dominant end products were acetate and hydrogen, usually in the molar ratio of 1.0:1.5. The strain degraded xylose and most of the hexoses and disaccharides tested as well as starch, pyruvate and the amino acids serine and threonine. No end product formation (above controls) was observed on arabinose, rhamnose, raffinose, glycerol, xylan or any of the cellulose substrate tested. The substrate spectrum has not been systematically investigated for *Thermoanerobacter* species but all species degrade various carbohydrates. Strain CMC_{15} is most closely related to is *T. yonseiensis T. keratinophilus, T. subterraneus* and *T. tengcongensis*. These four strains seem to have similar substrate spectrum as CMC_{15}, e.g. do not degrade arabinose, raffinose and sucrose (except for *T. yonseiensis* which degrades sucrose) (Fardeau et al. 2000; Kim et al. 2001; Xue et al. 2001; Riessen & Antranikian G). Some of the other species within the genus, e.g. *T. italicus, T. mathranii* and *T. sulfurophilus* have even more broad substrate spectrum and can also degrade arabinose, raffinose and sucrose (Kozianowski et al. 1997; Larsen et al. 1997; Bonch-Osmolovskaya et al. 1997).

Effect of initial glucose loadings on hydrogen production

From the kinetic experimental data (Figure 2) incomplete glucose degradation occurred from relatively low (20 mM; 3.6 g L^{-1}) concentrations and the pH dropped below 5.0. This inhibition may be of great significance in up-scaling batch processes and was investigated further by both increasing and decreasing the initial glucose concentrations. Lowering the initial glucose concentrations below 10 mM resulted in complete sugar degradation (Figure 4). However, raising the glucose from 10 mM to 20 mM led to incomplete degradation. Above 20 mM no further increase in product formation or lowering in pH was seen. It is a well-known phenomenon that high initial substrate concentrations may be inhibitory for thermophilic bacteria (van Ginkel and Sung 2001; Almarsdottir et al. 2010; Almarsdottir 2012). Inhibition by formation of end products is, however, more likely to be the main factor for this. The buffer capacity in the batch cultures used was 30 mM and the accumulation of acetate results in a pH drop and inhibition of substrate fermentation. Further experiments to investigate this further need to be performed by using e.g. combination of sugars (glucose and xylose) and increased phosphate buffer capacity in order to see if it is the low pH or high concentration of end products that causes the fermentation to stop.

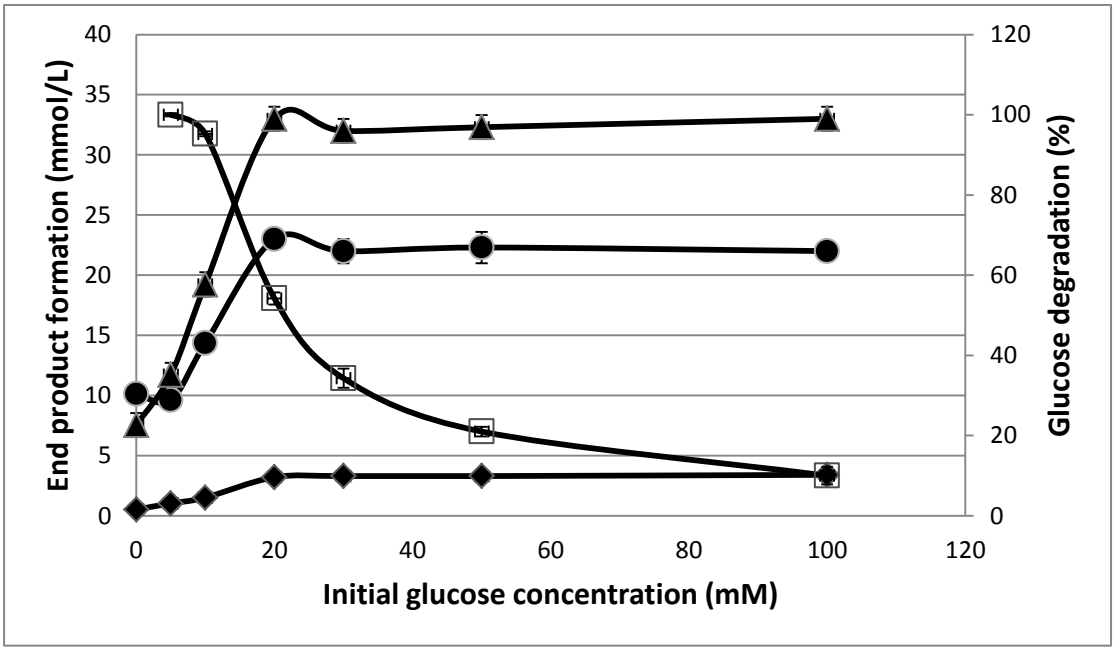

Figure 4. End product formation using different initial glucose concentrations. Also shown are percent of glucose degraded. Values represent means of two replicates and standard deviation are shown as error bars. Glucose (□), Ethanol (♦), Acetate (●), Hydrogen (▲).

The effect of partial pressure of hydrogen on end product formation

All the experiments above were performed in serum bottles supplemented with BM medium to give liquid – gas (L-G) ratio between 0.69 and 1.0. Ethanol production occurs in all experiments although less than acetate and hydrogen. It has been reported that the change in partial pressure of hydrogen affects end product formation of anaerobic bacteria. Thus at high pH_2 the product formation is shifted from acetate and H_2 towards more reduced products like ethanol and lactate (Nath & Das 2004; Hawkes et al. 2002; van Niel et al. 2006; Almarsdottir et al. 2010). This was tested in present study by culturing the strain on low glucose concentrations (10 mM) in 117.5 mL serum bottles with three different liquid to gas ratios (0.04, 1.04 and 3.27). Theoretical yields of hydrogen production is 4 moles of hydrogen per mole of hexose degraded if acetate is the only carbon containing_volatile end product (Nandi & Sengupta 1998; Hawkes et al. 2002). By using the fermentation data from end product formation obtained the following equations are observed (background values from yeast extract were subtracted):

1.0 Glucose → 1.65 Acetate + 0.12 EtOH + 3.10 H_2 + 1.77 CO_2 (L-G = 0.04; Eq.1)

1.0 Glucose → 1.33 Acetate + 0.33 EtOH + 2.10 H_2 + 1.77 CO_2 (L-G = 1.04; Eq.2)

1.0 Glucose → 1.10 Acetate + 0.57 EtOH + 1.70 H_2 1.62 CO_2 (L-G = 3.27; Eq.2)

In all three cases the glucose was completely degraded. The strain produces maximum 3.1 mol H_2 mol glucose^{-1} at very low partial pressure of hydrogen. By increasing the L-G ratio hydrogen yields drop to 1.7 mol and ethanol prodution increases almost five times. Various values of hydrogen production yields with thermophilic bacteria are available in literature. The highest yields reported are 4 moles of hydrogen (= theoretical maximum) from one mole of glucose by *Thermoanaerobacter tengcongensis* (Soboh et al. 2004) and by *Thermotoga maritima* (Schröder et al. 1994) under continuous hydrogen removal or at extremely low substrate concentration, respectively. High yields have also been observed for other extremophilic bacteria e.g. *Thermotoga* species but less by moderate thermophiles e.g. *Thermoanaerobacterium* and *Clostridium* species (Table 1).

Table 1. H_2 production from sugars by pure cultures of thermophilic bacteria in batch cultures. Hydrogen yields as well as substrate concentrations and incubation temperature are also shown. N/A = not analysed.

	Biomass	Substrate conc. (g L^{-1})	Hydrogen Yield (mmol / g sugar)	Temp (°C)	Reference
Thermotoga neopolitana	Glucose	2.5	3.9	77	Munro et al. (2009)
Thermotoga neopolitana	Glucose	7.0	3.2	77	Nguyen et al. (2010)
Thermotoga neopolitana	Glucose	5.0	N/A	70	Van Ooteghem et al. (2002)
Thermotoga maritima	Glucose	0.1	4.0	80	Schroder et al. (1994)
Thermoanaerobacter tengcongensis	Glucose	4.5	4.0	75	Soboh et al. (2004)
Caldicellulosiruptor saccharolyticus	Glucose	1.7	2.5	70	Kadar et al. (2004)
Caldicellulosiruptor saccharolyticus	Xylose	1.6	2.7	70	Kadar et al. (2004)
Thermoanaerobacterium thermosaccharolyticum	Glucose	10.0	2.4	60	Ren et al. (2008)
Thermoanaerobacterium thermosaccharolyticum	Xylose	10.0	2.2	60	Ren et al. (2008)
Clostrdium AK14	Glucose	3.6	2.2	50	Almarsdottir et al. (2010)
Thermoanaerobacter CMC15	Glucose	3.6	3.1	65	This study

Fermentation of hydrolysates from lignocellulosic biomass

Hydrogen production from lignocellulosic biomass has got increased attention recently. In general hydrogen yields from mesophilic and moderate thermophilic bacteria are lower as compared to the extremophilic bacteria (Table 2) for thermodynamic reasons. At high temperatures, the influence of the partial pressure of H_2 is less on the key enzymes responsible for H_2 production. For example, *Clostridium* AK_{14} , a moderate thermophile (T_{opt} = 45-50°C) that was investigated at our laboratory, shows a maximum of 2.6 mol H_2 mol glucose^{-1} with a typical acetate/butyrate production profile (Almarsdottir et al. 2010). Yields on hemp stem and barley straw hydrolysates were however much lower or between 0.6 – 0.8 mmol H_2 mol glucose^{-1} equivalent but up to 1.2 on cellulose (Table 2).

Table 2. H_2 production from batch cultures of agricultural wastes and energy crops. Volumetric H_2 production rates, H_2 yields as well as substrate concentrations and incubation temperature are also shown.

Culture	Biomass	Biomasse conc. (g L^{-1})	Hydrogen Yield (mmol g $sugar^{-1}$)	Temp (°C)	Reference
Caldicellulosiruptor. saccharolyticus	Paper sludge	8.4	3.7	70	Kadar et al. (2004)
Clostridium thermocellum	Whatman paper	0.1-4.5	0.8-1.9	60	Levin et al. (2006)
Thermotoga neopolitana	Cellulose	5.0	1.0-2.2	80	Nguyen et al. (2008)
Clostridium thermocellum	Barley straw	5.0	1.2	60	Magnusson et al (2008)
Thermoanaerobacterium thermosaccharolyticum	Corn stover	6.4-12.2	2.2	60	Cao et al. (2009)
Thermoanaerobacterium thermosaccharolyticum	Miscanthus	10.0	3.4	72	Vrije et al (2009)
Thermotoga neopolitana	Miscanthus	14.0	3.2	80	Vrije et al (2009)
Caldicellulosiruptor. saccharolyticus	Wheat straw	10.0	3.8	70	Ivanova et al. (2009)
Thermoanaerobacterium thermosaccharolyticum	Corn stover	10.0	2.7	60	Ren et al. (2010)
Clostrdium AK14	Cellulose	5.0	1.1-1.2	50	Almarsdottir et al. (2010)
Clostrdium AK14	Hemp stem	5.0	0.6-0.7	50	Almarsdottir et al. (2010)
Clostrdium AK14	Barley straw	5.0	0.7-0.8	50	Almarsdottir et al. (2010)
Thermoanaerobacter CMC15	Cellulose	4.5	2.3	65	This study

Clostridium thermocellum shows similar hydrogen yields on Whatman paper and barley hull hydrolysates (Nguyen et al. 2008; Magnusson et al. 2008). Extremophilic bacteria, e.g. *Thermotoga neapolitana* show higher yields or up to 2.2 mol H_2 from microcrystalline cellulose (Nguyen et al. 2008).

High yields have also been observed on lignocellulosic biomass by *Thermoanaerobacterium thermosaccharolyticus*, *Thermotoga neopolitana* and *Caldicellulosiruptor* (Cao et al. 2009; Vrije et al. 2009; Ivanova et al. 2009; Ren et al. 2010) (Table 2). *Caldicellulosiruptor saccharolyticus* has been used for H_2 production from various biomass without chemical pretreatment in batch culture (Ivanova et al., 2009). From wheat straw, this strain produced 2.04 mmol H_2 g $(dw)^{-1}$ (0.36 mol H_2 mol $glucose^{-1}$), i.e. less than 10% of theoretical yields of sugars present in the biomass. Yields calculated on the basis of glucose consumed were however extremely high, or 3.8 mol H_2 mol $glucose^{-1}$.

Strain CMC_{15} was inoculated in BM medium containing 4.5 g L^{-1} of hydrolysates from different types of complex biomass (cellulose, hemp leafs, hemp stem fibers, newspaper, barley straw and grass) (Figure 5).

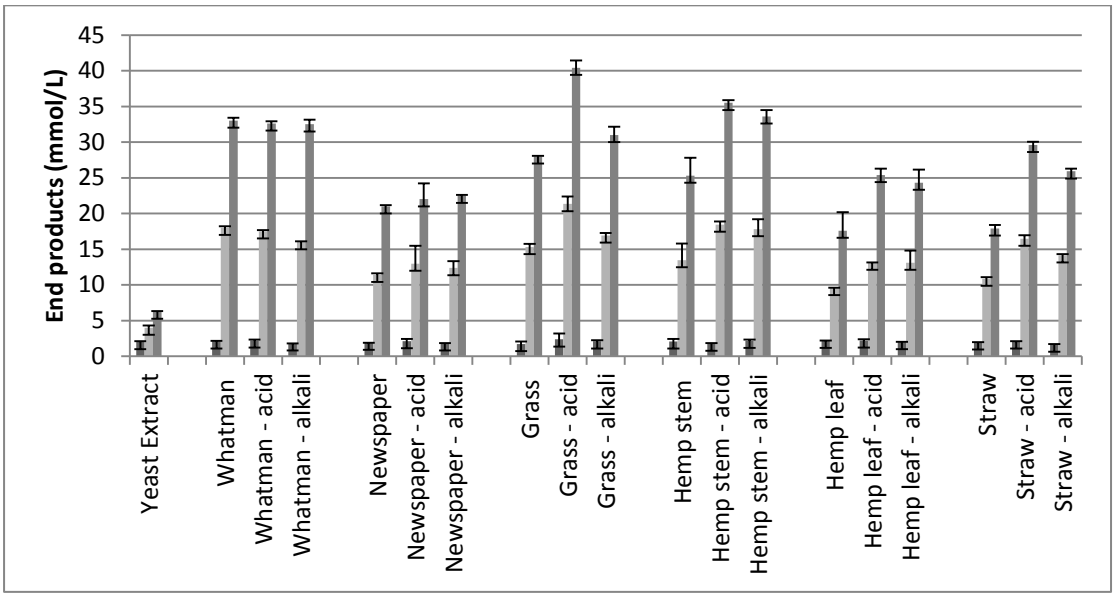

Figure 5. End product formation from hydrolysates (4.5 g L^{-1}) from different biomass. From left to right; ethanol, acetate and hydrogen. Data represents average of two replicate experiments. Standard deviations are shown as error bars.

Glucose was analyzed in the hydrolysate after enzymatic hydrolysis and chemical pretreatment. The Whatman paper used in the experiment is 99% pure and total hydrolysis should result in the formation of 27.5 mM of glucose. Slightly lower values were however analysed, or between 23.6 to 25.4 mM. After fermentation more than half of the glucose remained in the culture broth of Whatman paper hydrolysates. Hydrogen yields were 5.8 to 6.0 mol-H_2 g cellulose^{-1} but 14.4 mol H_2 g^{-1} glucose equivalents degraded (2.33 mol H_2 mol glucose). This experiment was done in serum bottles with L-G ratio of 0.69 and are in agreement with pH_2 experimental data shown earlier. Similar results were observed between chemically pretreated cellulose and enzymatically pretreated cellulose. This similarity in end product formation was also observed for newspaper hydrolysate with and without chemical pretreatment. The four types of lignocellulosic biomass, however, showed lower yields with only enzymatic pretreated biomass as compared with either acid or alkali pretreated substrates. The lowest yields for untreated lignocellulosic biomass were observed

from hemp leaves, 2.52 mM-H_2 g^{-1}, and highest on grass hydrolysates, 4.83 mM-H_2 g^{-1}. These yields were, however, substantially increased with chemical pretreatment, most profoundly on alkali pretreated straw where yields were almost two times higher as compared with enzymatically pretreated straw. Highest yields obtained were on acid pretreated grass or 7.6 mM-H_2 g^{-1}. *Thermoanaerobacter* species have not been studied to any extent concerning hydrogen production capacity on lignocellulosic biomass. The genus has got more attention because of ethanol production potential of many species, e.g. *T. ethanolicus, T. thermohydrosulfuricus* and other (Georgieva et al. 2008).

For comparison, the same experimental set up was done for the extremophile *Caldicellulosiruptor saccharolyticus* (DSM 8903), a well known hydrogen producer. Fermentation of glucose (20 mM) by the strain showed the following stoichiometry:

1.0 Glucose → 1.40 Acetate + 0.05 EtOH + 2.4 H_2 + 1.45 CO_2

Hydrogen yields from cellulose hydrolysates were 10.9 mol-H_2 g TS^{-1} of cellulose or 43% higher as compared to strain CMC_{15} (Figures 5 and 6).

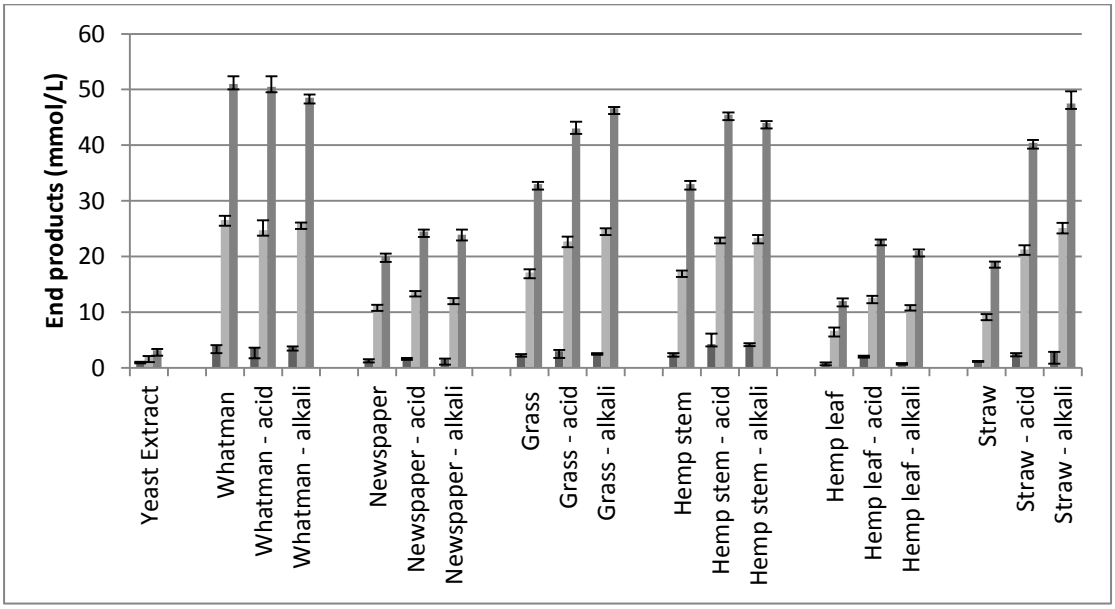

Figure 6. End product formation from hydrolysates by Caldicellulosiruptor saccharolyticum (4.5 g L^{-1}) from different biomass. From left to right; ethanol, acetate and hydrogen. Data represents average of two replicate experiments. Standard deviations are shown as error bars.

Also, higher hydrogen yields were observed on other types of biomass. Glucose was unfortunately not analyzed in these experiments and higher yields on cellulose could be because the strain is less sensible for initial glucose concentrations as compared with CMC_{15}. Lactate has also been reported to be produced by *Caldicellulosiruptor* (Zeidan and van Niel, 2010) but was not analysed in our study.

Conclusion

Hydrogen production was studied by *Thermoanaerobacter* CMC_{15} isolated from a hot spring in Iceland. The main aim of the study was to investigate the hydrogen production potential of the strain, both from simple sugars and various lignocellulosic biomasses. The strain produced maximally 3.1 mol H_2 mol glucose under very low partial pressure of hydrogen (0.8 kPa). Highest hydrogen yields from lignocellulosic biomass were observed on grass hydrolysate pretreated with acid, 7.6 mol H_2 g grass. In general chemical pretreatment of lignocellulosic biomass was very important for the hydrogen yields. Studies of the effect of various environmental factors revealed that the biggest drawback of the strain is its extreme sensitivity towards either initial substrate concentration or accumulation of acetate that lowers the pH and stops glucose fermentation. This needs to be investigated in more detail for the strain.

Acknowledgements

This work was sponsored by RANNÍS, (Technology Development Fund, projects 081303408 (BioEthanol) and RAN091016-2376 (BioFuel), The Nordic Energy Fund (NER; project BioH2, 06-Hydr-C13), The Research Fund of the University of Akureyri.

References

Almarsdottir AR, Taraceviz A, Gunnarsson I, Orlygsson J. 2010. Hydrogen production from sugars and complex biomass by *Clostridium* species, AK14, isolated from Icelandic hot spring. Icelandic Agric. Sci. 23:61-71.

Almarsdottir AR, Sigurbjornsdottir MA, Orlygsson J. 2012. Effect of various factors on ethanol yields from lignocellulosic biomass by *Thermoanaerobacterium* AK17. Biotechnol. Bioeng. 109:686-694.

Balat M, Balat H, Öz C. 2008. Process in bioethanol processing. Prog. Energ. Combust. 34:551-573.

Bonch-Osmolovskaya, EA, Miroshnichenko ML, Chernykh NA, Kostrikina NA, Pikuta EV, Rainey FA. 1997. Reduction of sulfur by moderately thermophilic organotrophic bacteria and the description of *Thermoanaerobacter sulfurophilus* sp. nov. Microbiology. 5:483-489.

Cao G-L, Ren N, Wang A, Lee D, Guo W, Liu B, Feng Y, Zhao, Q. 2009. Acid hydrolysis of corn stover for biohydrogen production using *Thermoanaerobacterium thermosaccharolyticum* W16. Int. J. Hydrogen Energ. 34:7182-7188.

Collins MD, Lawson PA, Willems A, Cordoba JJ, Garayzabal FJ, Garcia P, Cai, J, Hippe H, Farroe JAE. 1994. The phylogeny of the genus *Clostridium* – Proposal of 5 new genera and 11 new species combinations. Int. J. Syst. Bacteriol. 44:812-826.

de Vrije T, Bakker RR, Budde MAW, Lai MH, Mars AE, Claassen, PAM. 2009. Efficient hydrogen production from the lignocellulosic energy crop miscanthus by the extreme thermophilic bacteria *Caldicellulosiruptor saccharolyticus* and *Thermotoga neapolitana*. Biotechnol. Biofuel. 2:12.

Fardeau M-L, Patel B.K.C, Magot M & Ollivier B. 1997. Utilization of serine, leucine, isoleucine, and valine by *Thermoanaerobacter brockii* in the presence of thiosulfate or *Methanobacterium* sp. as electron acceptors. Anaerobe. 3:405-410.

Fardeau M-L, Magot M, Patel BKC, Thomas P, Garcia J-L, Ollivier B. 2000. *Thermoanerobacter subterraneus* sp. nov., a novel thermophile isolated from oilfiled water. Int. J. Syst. Evol. Micr. 50:2141-2149.

Fardeau M-L, Bonilla Salinas M, L'Haridon S, Jeanthon V, Verhé F, Cayol JL, Patel BKC, Garcia JL, Ollivier B. 2004. Isolation from oil reservoirs of novel thermophilic anaerobes phyogenetically related to *Thermoanaerobacter subterraneus*: reassignment of *T. subterraneus*, *Thermoanaerobacter yonseiensis*, *Thermoanaerobacter tengcongensis* and *Carboxydibrachimum pacifium* to *Caldanaerobacter subterraneous* gen.nov., sp.nov., comb. Nov. as four novel subsepcies. Int. J. Syst. Evol. Micr. 54:467-474.

Georgieva TI, Mikkelsen MJ, Ahring BK. 2008. Ethanol production from wet-exploded wheat straw hydrolysate by thermophilic anaerobic bacterium

Thermoanaerobacter BG1L1 in a continuous immobilized reactor. Appl. Biochem. Biotech. 145:99-110.

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

Hallenbeck PC, Benemann JR. 2002. Biological hydrogen production; fundamentals and limiting processes. Int. J. Hydrogen Energ. 27:1185-1193.

Hawkes FR, Dinsdale R, Hawkes DL, Hussy I. 2002. Sustainable fermentative hydrogen production: challenges for process optimisation. Int. J. Hydrogen Energ. 27:1339-1347.

Jones P. 2008. Improving fermentative biomass-derived H2-production by engineered microbial metabolism. Int. J. Hydrogen Energ. 33: 5122-5130.

Kádár, Z, de Vrijek T, van Noorden GE, Budde MAW, Szengyel Z, Reczey K. 2004. Yields from glucose, xylose, and paper sludge hydrolysate during hydrogen production by the extreme thermophile *Caldicellulosiruptor saccharolyticus*. Appl. Biochem. Biotechnol. 113: 497-508.

Kapdan IK., Kargi F. 2006. Bio-hydrogen production from waste materials. Enzyme Microb Technol. 38:569-582.

Kataoka N, Miya A, Kiriyama K. 1997. Studies on hydrogen production by continuous culture system of hydrogen-producing anaerobic bacteria. Water Sci. Technol. 36:41-47.

Kim B-C, Grote R, Lee D-W, Antranikian G, Pyun Y-R. 2001. *Thermoanaerobacter yonseiensis* sp. nov., a novel extremely thermophilic, xylose utilizing bacterium that grows at up to 85°C. Int. J. Syst. Evol. Micr. 51:1539-1548.

Koskinen PEP, Lay CH, Beck SR, Tolvanen KE, Koksonen AH, Orlygsson J, Lin CY, Puhakka JA, 2008a. Bioprospecting thermophilic microorganisms from Icelandic hot springs for hydrogen and ethanol production. Energ. Fuel. 22:134-140.

Koskinen PE, Lay CH, Puhakka JA, Lin PJ, Wu SY, Orlygsson J, Lin CY, 2008b. High efficiency hydrogen production by an anaerobic, thermophilic enrichment culture from Icelandic hot spring. Biotechnol. Bioeng. 101:665-678.

Kozianowski G, Canganella F, Rainey FA, Hippe H, Anthranikian G. 1997. Purification and characterization of thermostable pectate-lyase from a newly isolated thermophilic bacterium, *Thermoanerobacter italicus* sp. nov. Extremophiles. 1:171-182.

Lee YE, Jain MK, Lee C, Lowe SE, Zeikus JG. 1993. Taxonomic distinction of saccharolytic thermophilic anaerobes: Description of *Thermoanaerobacterium xylanolyticum* gen. nov., sp. nov., and *Thermoanaerobacterium saccharolyticum* gen. nov., sp. nov.; reclassification of *Thermoanaerobium brockii*, *Clostridium thermosulfurogenes*, and *Clostridium thermohydrosulfuricum* E100-69 as *Thermoanaerobacter brockii* comb. nov., *Thermoanaerobacterium thermosulfurigenes* comb. nov., and *Thermoanaerobacter thermohydrosulfuricus* comb. nov., respectively; and

transfer of *Clostridium thermohydrosulfuricum* 39E to *Thermoanaerobacter ethanolicus*. Int. J. Syst. Bacteriol. 43: 41-51.

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

Laurentin A, Edwards CA. 2003. A microtiter modification of the anthrone-sulfuric acid colorimetric assay for glucose-based carbohydrates. Anal. Biochem. 315:143-145.

Levin DB, Lawrence P, Love M. 2004. Biohydrogen production: prospects and limitations to practical application. Int. J. Hydrogen Energ. 29:173-185.

Levin DB, Islam R, Cicek N, Sparling R. 2006. Hydrogen production by Clostridium *thermocellum* 27405 from cellulosic biomass substrates. Int. J. Hydrogen Energ. 31:1496-1503.

Magnusson L, Islam R, Sparling R, Levin D, Cicek N. 2008. Direct hydrogen production from cellulosic waste materials with a single-step dark fermentation process. Int. J. Hydrogen Energ. 33:5398-5403.

Munro SA, Zinder SH, Walker LP. 2009. The fermentation stoichiometry of *Thermotoga neapolitana* and influence of temperature, oxygen, and pH on hydrogen production. Biotechnol. Progr. 25:1035-1042.

Nandi R, Sengupta S. 1998. Microbial production of hydrogen: An overview. Crit. Rev. Microbiol. 24:61-84.

Nath K, Das D. 2004. Improvement of fermentative hydrogen production: Various approaches. Appl. Microbiol. Biotech. 65:520-529.

Nguyen TD, Han SJ, Kim JP, Kim MS, Oh YK, Sim SJ. 2008. Hydrogen production by the hyperthermophilic eubacterium, *Thermotoga neapolitana*, using cellulose pretreated by ionic liquid. Int. J. Hydrogen Energ. 33:5161-5168.

Nguyen TD, Han SJ, Kim JP, Kim MS, Sim SJ. 2010. Hydrogen production of the hyperthermophilic eubacterium, *Thermotoga neapolitana* under N-2 sparging condition. Bioresource Technol. 101:S38-S41.

Oh Y-K, Seol EH, Kim JR, Park S. 2003. Fermentative biohydrogen production by a new chemoheterotrophic bacterium *Citrobacter* sp Y19. Int. J. Hydrogen Energ. 28:1353-1359.

Orlygsson J, Baldursson SRB. 2007. Phylogenetic and physiological studies of four hydrogen-producing thermoanaerobes from Icelandic geothermal areas. Icelandic Agric. Sci. 20:93-106.

Ren N, Cao G, Guo W, Wang A, Zhu Y, Liu B, Xu J-F. 2010. Biological hydrogen production from corn stover by moderately thermophile *Thermoanaerobacterium thermosaccharolyticum* W16. Int. J. Hydrogen Energ. 35:2708-2712.

Ren N, Cao G, Wang A. Lee D, Guo W, Zhu Y. 2008. Dark fermentation of xylose and glucose mix using isolated *Thermoanaerobacterium thermosaccharolyticum* W16. Int. J. Hydrogen Energ. 33:6124-6132.

Renewable Fuels Association. 2011. Choose ethanol. 16. mars retrieved from http://chooseethanol.com/what-is-ethanol/entry/ethanol-at-a-glance/

Riessen S, Antranikian G. 2001. Isolation of *Thermoanerobacter keratinophilus* sp. nov., a novel thermophilic, anaerobic bacterium with keratinolytic activity. Extremophiles. 5:399-408.

Sánchez ÓJ, Cardona CA. 2008. Trends in biotechnological production of fuel ethanol from different feedstocks. Bioresource Technol. 13:5270-5295.

Saxena RC, Adhikari DK, Goyal HB. 2009. Biomass-based energy fuel through biochemical routes: A review. Renew. Sust. Energ. Rev. 13:167-178.

Schroder C, Selig M, Schonheit P. 1994. Glucose fermentation to acetate, CO2 and H_2 in the anaerobic hyperthermophilic eubacterium *Thermotoga maritima* - involvement of the Embden-Meyerhof pathway. Arch. Microbiol. 161:460-470.

Sigurbjornsdottir MA, Orlygsson J. 2012. Combined hydrogen and ethanol production from sugars and lignocellulosic biomass by *Thermoanaerobacterium* AK54. Appl. Energ. Doi:10/1016/J.apenergy.2011.11.035.

Soboh B, Linder D, Hedderich R. 2004. A multisubunit membrane-bound [NiFe] hydrogenase and an NADH-dependent Fe-only hydrogenase in the fermenting bacterium *Thermoanaerobacter tengcongensis*. Microbiology. 150:2451-2461.

Sveinsdottir M, Baldursson SRB, Orlygsson J. 2009. Ethanol production from monosugars and lignocellulosic biomass by thermophilic bacteria isolated from Icelandic hot springs. Icelandic Agric. Sci. 22:45-58.

Taguchi F, Chang JD, Takiguchi S, Morimoto M. 1992. Efficient hydrogen production from starch by a bacterium isolated from termites. J. Ferment. Bioeng. 73:244-245.

Taylor MP, Eley KL, Martin S, Tuffin M, Burton SG, Cowan DA. 2008. Thermophilic ethanologenesis: future prospects for second-generation bioethanol production. Trends Biotechnol. 27: 398-405.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25:4876-4882.

Turner JA. 2004. Sustainable hydrogen production. Science. 305:972-974.

van Ginkel S, Sung S. 2001. Biohydrogen production as a function of pH and substrate concentration. Environ. Sci. Technol. 35:4726-4730.

van Groenestijn JW, Hazewinkel JH, Nienoord M, Bussmann PJ. 2002. Energy aspects of biological hydrogen production in high rate bioreactors operated in the thermophilic temperature range. Int. J. Hydrogen Energ. 27:1141-1147.

van Niel EW, Budde MA, de Haas GG, van der Wal FJ, Claassen PA, Stams AJ. 2002. Distinctive properties of high hydrogen producing extreme thermophiles, *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii*. Int. J. Hydrogen Energ. 27:1391-1398.

van Niel EWJ, Claassen PAM, Stam AJM. 2006. Substrate and end product inhibiton of hydrogen production by the extreme thermophile, *Caldicellulosiruptor saccharolyticus*. Biotechnol. Bioeng. 121:515-533.

van Ooteghem SA, Beer SK, Yue PC. 2002. Hydrogen production by the thermophilic bacterium *Thermotoga neapolitana*. Appl. Biochem. Biotech. 98-100:177-189

Xue Y, Xu Y, Liu Y, Ma Y, Zhou P. 2001. *Thermoanerobacter tengcongensis* sp. nov., a novel anaerobic, saccharolytic, thermophilic bacterium isolated from a hot spring in Tengcong, China. Int. J. Syst. Evol. Micr. 51:1335-1341.

Yokoi H, Ohkawara T, Hayashi S, Takasaki Y. 1995. Characteristics of hydrogen production by aciduric *Enterobacter aerogenes* strain HO-39. J. Ferment. Bioeng. 80:571-574.

Zaldivar J, Nielsen J, Olsson L. 2001. Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. Appl. Microbiol. Biot. 56:17-34.

Zeidan AA. & van Niel EWJ. 2010. A quantitative analysis of hydrogen production efficiency of the extreme thermophile *Caldicellulosiruptor owensensis* OLT. Int. J. Hydrogen Energ. 35:1128-1137.

6 Conclusions

The present study included the production of biohydrogen and bioethanol by two thermophilic bacteria strains (AK_5 and CMC_{15}), isolated from Grensdalur (SW-Iceland). Both strains were examined regarding their ability to degrade various carbon substrates present in complex biomass and produce ethanol and hydrogen.

Various physiological experiments were performed on the strains to determine their optimal growth conditions, substrate utilization spectrum effects of i.e.the effect of initial glucose concentration and partial pressure of hydrogen on end product formation. Additionally the effects of electron scavenging systems (thiosulfate and hydrogenotrophic methanogen) on the end product formation of strain AK_5 was examined. The kinetics of glucose degradation and end product formation was registered. Partial 16S rRNA analysis revealed that both strains belong to the genera *Thermoanaerobacter*. Strain AK_5 has 99.1% homology to *Thermoanaerobacter thermohydrosulfuricus* and strain CMC_{15} has 99.9% homology to *Thermoanaerobacter yonseiensis*.

Thermoanaerobacter AK_5 has a broad substrate spectrum degrading various hexoses, pentoses, disaccharides and polymeric substrates primarily to ethanol but also acetate, H_2 and CO_2. Ethanol yields on glucose and xylose were 1.55 and 1.35 mol EtOH mol glucose^{-1} respectively and it produces up to 7.7 mM EtOH g^{-1} cellulose and 4.4 mM g^{-1} grass hydrolysate. The strain is highly flexible for either ethanol or acetate production depending on the culture conditions used. When cultivated under electron scavenging systems it shifts almost completely away from ethanol production and acetate becomes its main end product. The main drawback of the strain is its inhibition of substrate (glucose) utilization at elevated substrate concentrations. This limitation could however be solved by cultivating the strain in fed batch or continuous mode.

Thermoanaerobacter CMC_{15} has similar substrate spectrum as strain AK_5 but the main end product formation is towards acetate and hydrogen instead of ethanol. Its maximal hydrogen yields, achieved at a very low partial pressure of hydrogen (0.8 kPa), were 3.1 mol H_2 mol glucose^{-1}. Highest hydrogen yields from lignocellulosic

biomass was observed on grass hydrolysate pretreated with 0.50% (w/v) acid; 4.83 mol H_2 g^{-1} grass. The strain is however extremely sensitive to either initial substrate concentration or accumulation of acetate that lowers the pH and stops glucose fermentation.

Both *Thermoanaerobacter* AK_5 and *Thermoanaerobacter* CMC_{15} are promising ethanol and hydrogen producers, respectively, with high yields from lignocellulosic biomass. The ethanol yields of *Thermoanaerobacter* AK_5 can be maximized by cultivating the strain under high pH_2 hydrogen and *Thermoanaerobacter* CMC_{15} hydrogen yields are desirable as long as the initial substrate concentration is kept low or the pH of the culture is kept above 5. This will be investigated in more detail for the strain.