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Biofuel Production from Lignocellulosic Biomass by Thermophilic Bacteria

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June 2013

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Master thesis for 90 credit M.Sc. in Biotechnology

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Útdráttur

Rannsóknin gekk út á framleiðslu etanóls og vetnis með hitakærum bakteríum í lokuðum ræktum. Tveir stofnar voru valdið með tilliti til etanólframleiðslu, stofnar J1 og J4, og tveir með tilliti til vetnisframleiðslu, stofnar J3 og J4.

Stofnar J1 og J4 tilheyra ætthvíslinni *Thermoanaerobacter* og teljast báðir hitakærir. Stofn J1 er mjög góður etanólframleiðandi, þolir háa upphafsstyrki hvarfefna og er hvorki viðkvæmur fyrir mismunandi hlutþrýsting vetnis eða utanaðkomandi rafeindaþegum. Hann framleiðir 1.7 mól af etanóli á hvert mól glúkósa. Hæsta framleiðsla stofnsins á hýdrólýsötum reyndist vera 6.82 mM etanól á gramm af Whatman pappír. Báðir stofnarnir reyndust sérlega hitaþolnir og gátu brotið niður fjölbreytt úrval hvarfefna. J1 gat vaxið í viðurvist 4.2% etanólstyrks, en J4 þoldi 3.4%. Stofn J4 er góður vetnisframleiðandi en er aftur á móti viðkvæmur gagnvart háum upphafsstyrk hvarfefna. Lokaafurðir stofnsins eru aðallega ediksýra og vetni. Stofninn hneigist til etanólframleiðslu við háan hlutþrýsting vetnis. Stofninn framleiddi að hámarki tæplega 2.5 mól af vetni á mól af glúkósa og 20.5 mmol/L á hálmi formeðhöndluðum með basa.

Hinir tveir stofnarnir eru miðlungshitakærir og tilheyra annars vegar ættkvíslinni *Paenibacillus* (J2) og hins vegar *Clostridium* (J3). J2 er góður etanólframleiðandi en J3 góður vetnisframleiðandi. Báðir stofnarnir geta brotið niður fjölbreytt úrval hvarfefna. Stofn J2 framleiddi 1,5 mól af etanóli á mól glúkósa og 23.6 mM á óformeðhöndluðum Whatman pappír, en mesta framleiðslan á flóknum lífmassa var 14.6 mM á sýruformeðhöndluðu grasi. J2 gat vaxið við 3.4% etanólstyrk. Stofn J3 notast við hefðbundið ediksýru/smjörsýru gerjunarmynstur. Ekki reyndist mögulegt að nota stofninn við allar tilraunir rannsóknarinnar sökum erfiðleika við uppræktun hans. Bæði J2 og J3 eru viðkvæmir fyrir háum upphafsstyrk hvarfefna.

Stofnar J1 og J4 voru notaðir í ítarlegri hýdrólýsattilraun á vallarfoxgrasi þar sem áhrif bæði mismunandi upphafsstyrks hvarfefna sem og mismunandi hlutþrýstings vetnis voru rannsökuð. Stofn J1 braut niður svo gott sem allar sykrurnar í hýdrólýsatinu og varð hvorki fyrir hindrun vegna hás upphafsstyrks hvarfefna né hás hlutþrýstings vetnis. Stofn J4 varð aftur á móti fyrir hindrun af báðum þessum þáttum.

Abstract

This investigation was on the production of ethanol and hydrogen by thermophilic bacteria in batch cultures. Two strains were selected for study of ethanol, strains J1 and J2, and two for hydrogen production, strains J3 and J4.

Strains J1 and J4 belong to the genus *Thermoanaerobacter* and are highly thermophilic. Strain J1 is highly ethanologenic, tolerates high substrate loadings, and is not affected by different partial pressure of hydrogen or the presence of hydrogen scavenging systems in batch cultures. It produces 1.7 mol ethanol per mol glucose. Maximum production from various types of hydrolysates was 6.82 mM per g of Whatman paper. Both strains were extremely heat tolerant and possess a broad substrate spectrum. J1 tolerated ethanol concentrations up to 4.2% and J4 up to 3.4%. Strain J4 is a good hydrogen producer but is highly sensitive to moderate substrate loadings, and produces mostly acetate and hydrogen from various carbohydrates. Under high pH_2 the strain became ethanolgenic. The strain produced maximally almost 2.5 mol hydrogen per mol glucose and 20.5 mmol/L on alkali pretreated straw.

The other two strains were moderate thermophiles and belong to *Paenibacillus* (J2) and *Clostridium* (J3) and were good ethanol and hydrogen producers, respectively. They both possess a broad substrate spectrum. Strain J2 produced 1.5 mol ethanol per mol glucose and 23.6 mM on unpretreated Whatman paper, but the highest yields on complex biomass was 14.6 mM on acid pretreated grass. J2 tolerated 3.4% ethanol concentration. The *Clostridium* strain J3 shows a classical acetate and butyrate fermentation pattern. Due to problems cultivating this strain, it was not possible to conduct all experiments on this strain. Both strains are sensitive to high substrate loadings.

Both strains J1 and J4 were subjected to detailed experiment on grass hydrolysates with different hydrolysate concentrations and different L/G ratios. Strain J1 degraded almost all sugars present in the hydrolysates and was not inhibited by neither increased hydrolysate loadings nor different partial pressure of hydrogen. Strain J4 was, however, severely inhibited by both factors.

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Jan Eric Jessen,
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1 Research objectives

The objective of this research was to screen the thermophilic, anaerobic bacteria culture collection of the University of Akureyri. These bacteria originate from hot springs in Iceland, where the main aim was to find potential ethanol and hydrogen producers. Four interesting strains were selected for further investigations such as phylogenic identification, ability to utilize various carbon sources, the effect of different culture conditions and tolerance to heat and ethanol concentrations.

The investigation had a great emphasis on biofuel production from lignocellulosic materials. All the selected strains were used in experiments where six different lignocellulosic hydrolysates, each pretreated both with dilute acid and alkali, were used as a carbon source. Furthermore, two similar types of Timothy grass (*Phleum pratense*) were analyzed at the Technical University of Denmark (DTU). Analyzed grass samples were used in another set of hydrolysate experiment for two of the strains. This provided the opportunity to investigate the effect of different initial concentrations of hydrolysate and different partial pressure of hydrogen where the initial sugar concentrations and chemical properties of the hydrolysates were known.

Results from experiments on one of the strains have been published in the Journal of Biomedicine and Biotechnology (Jessen & Orlygsson, 2012).

2 Introduction

One of the key issues of life is energy cycling between living organisms and the environment. The ability to use energy from the environment is essential for every single cell and enables it for instance to perform chemical reactions, grow, reproduce and maintain homeostasis (Silverthorn, 2010).

The three different forms of energy known are kinetic, potential, and internal energy. Kinetic energy is the energy of motion whereas potential energy is caused by the position of a system, e.g. in terms of a gravitional or electromagnetic field. Since internal energy is defined as the sum of all molecular, atomic and subatomic energies of matter (the total energy contained by a thermodynamic system), it cannot be measured precisely (Silverthorn, 2010; Doran, 2013).

The act of using sunlight to produce sugars and organic compounds by the process of photosynthesis is a conversion from one energy form to another. By this, plants store the energy in a chemical form called biomass. When performing physical activity, the human body converts potential energy to kinetic energy; glycogen is used to perform muscle contraction. The use of ATP to carry out chemical reactions is another form of energy conversion (Campbell & Reece, 2005).

Energy conversion is however not only restricted to the physiology of organisms. With their acts, they also have an impact on the environment regarding conversion of energy of one form to another and changing the energy condition of objects. In many cases, this does not seem to have a significant impact on the environment; a bird increasing the potential energy of dead straw, using it to construct a nest on a branch or a child converting the potential energy of a stone to kinetic energy by throwing it down from a cliff. On the other hand, these non-physiological acts can have a crucial impact on the environment. Since energy is needed to propel cars, ships and airplanes, heat houses and for industry and constructions, the human race has to consider the effects of different options regarding energy sources. The enormous scope of energy consumption is one of the greatest topics of the human community.

2.1 Sustainable development

In 1987, the United Nations World Commission on Environment and Development published *Our Common Future*, also known as the Brundtland Report, in which the concept of sustainable development is defined;

> Sustainable development is development that meets the needs of the present without compromising the ability of future generations to meet their own needs.

This definition is thoroughly clarified in the report and explained how the concept of sustainable development has to take note of social, economical and environmental factors (United Nations, 1987).

It is evident that the energy use of mankind is very far from being sustainable. The two main reasons are the impact of the combustion of petrochemicals or so-called "fossil fuels" (petroleum, coal, natural gas, etc.) on the environment and that fossil fuels are utilized faster than their generation time in nature. In the 20th century, the worldwide energy consumption increased 17-fold (Demirbas, 2008). Even though the use of fossil fuels has a much longer history, a general awareness concerning this issue only started a few decades ago in context with a dramatical increase of the scope of the problem.

One of the most important greenhouse gases is carbon dioxide, which is released in large amounts as a result of combustion of fossil fuel. In February 2013, the concentration of carbon dioxide in the atmosphere was 396 ppm, which is an increase of 7% from the turn of the century, and an increase of 24% in the last 50 years (Mauna Loa Observatory, 2013).

The increasing concentrations of greenhouse gases in the atmosphere is causing climate changes which can have a decisive effect on the earth's biota in near future. According to the Intergovernmental Panel on Climate Change (IPCC), the earth's average temperature rose by 0.74°C from 1906 to 2005. When the results were published, eleven of the last twelve years in the research (from 1995 to 2006) rank among the twelve warmest years since systematic global surface temperature measures began in 1850 (Mu & Mu, 2013). The global surface temperature for the last 133 years are shown in Figure 1. Global warming and accumulation of greenhouse gases is known to

warm the oceans, decrease Arctic sea ice and polar ice caps, and increase the number of extreme weather events. In the 20th century, global sea level rose by 17 centimeters, and the rate of sea level rising has increased in the last decades (NASA, 2013). Another consequence of increasing CO_2 levels in the atmosphere is an increased absorbance of this compound in the upper layer of the oceans, leading to substantial acidification (Sabine *et al.*, 2004). A 30% decrease in pH has been observed since the beginning of the Industrial Revolution (Mauna Loa Observatory, 2013).

Although an expected rise in energy demand in near future is not taken into consideration, continued climate change will be observed. The aforementioned IPCC report claims that even if the release of greenhouse gases would be constant to the levels in the year 2000, a further warming trend of about 0.1°C per decade would be observed in the next two decades (Mu & Mu, 2013). Both the severity of continuing climate changes and the need for an energy source which can serve the energy requirements of the future are widely accepted.

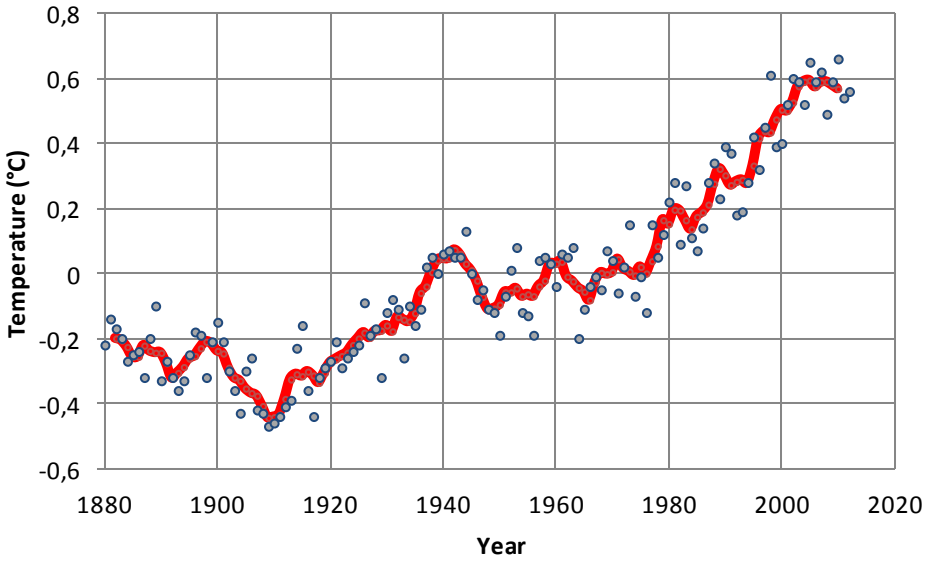

Figure 1. Average global surface temperature from 1880 to 2012. The gray dots represent the mean temperature for each year whereas the red line shows the development of average temperature over a 5 year period. Data from: (NASA, 2013)

2.2 Renewable energy

The essential replacement of fossil fuels by renewable energy sources is a both a slow and an expensive process. Renewable energy sources such as solar power, wind, hydroelectric, biomass and geothermal steam generation, are receiving increased interest. In recent years, about the half of the global new electricity capacity installed was derived from renewable sources. In 2011, 71% of energy sources installed in the European Union was renewable. In the same year, it was estimated that 17% of the worldwide energy consumption was from renewable energy sources (Figure 2) (REN21, 2012).

Many of the industrialized countries, which are responsible for the largest share of energy use and release of greenhouse gases, have already made long-term plans of increasing the proportion of renewable energy sources. One of the largest programs is running in Germany, where a scheme named *Energiewende* sets the goal to generate at least 35% of the country's electricity from renewable sources by 2020, and to increase the rate to over 80% by 2050. This is planned to be achieved by replacing fossil fuels with several renewable energy sources, including solar power, wind power and energy sources from biomass. Today, over 250 Terawatt-hours of electricity are produced in the country by combusting coals, but by 2050 coals should almost completely been taken out of use. Even though the emphasis on

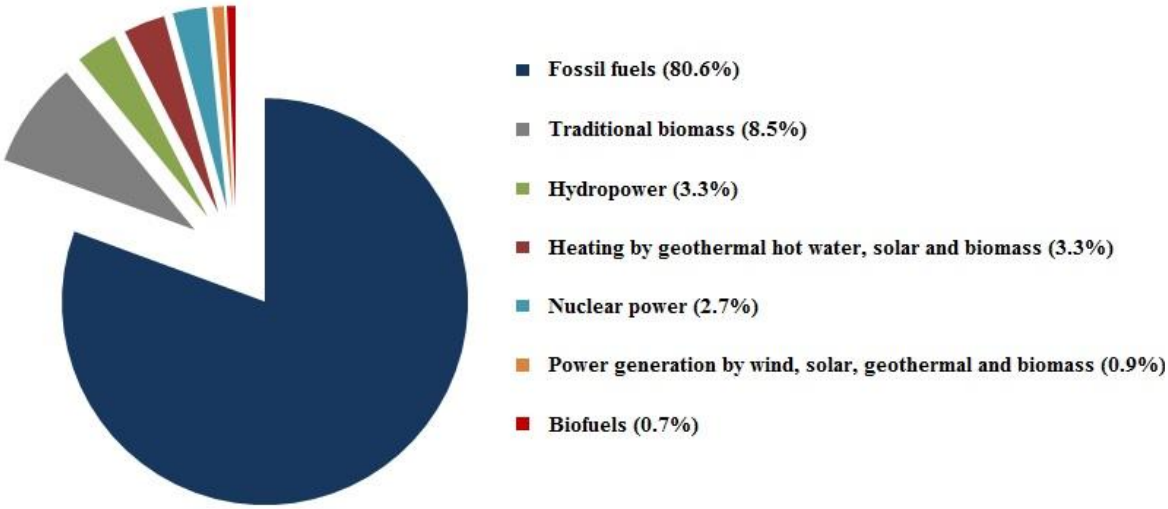

Figure 2. Shares of different energy sources of global energy consumption in 2010 (REN21, 2012).

investing in the development of renewable and environmental friendly energy sources is typically a political dispute, all the political parties of the German Parliament support the program (Schiermeier, 2013). The broad solidarity on solving this issue is of great importance and raises hopes concerning Germany's plan to be a model for a global replacing of fossil fuels by renewable energy sources.

In the United States, 11.8% of the primary energy production was from renewable sources in 2011, compared with 10.9% in 2010. 39% of the national electric capacity additions in 2011 were made up by renewable energy. The highest amount of renewable power capacity observed by any country in 2011 was in China; 282 GW. More than three quarters of this renewable energy was hydroelectric power. Over one third of the newly installed electric capacity was renewable, of which the majority was non-hydroelectric (REN21, 2012).

Table 1 sums up several different factors regarding the worldwide evolution towards renewable energy sources. If the values for the three years shown are compared, it is obvious that progress is being made. Certainly there is still a long road ahead, in the light of the fact that in 2010 still over 80% of the global energy consumption were fossil fuels (REN21, 2012). On the other hand, this should be considered as a long-term project and positive signs should be encouraging for keeping up the good work.

Table 1. Global values regarding renewable energy, in 2009-2011. Data from: (REN21, 2012).

	Unit	Year		
		2009	2010	2011
Investment in new renewable capacity (annual)	billion USD	161	220	257
Renewable power capacity (not including hydro)	GW	250	315	390
Renewable power capacity (including hydro)	GW	1170	1260	1360
Hydropower capacity (total)	GW	915	945	970
Solar PV capacity (total)	GW	23	40	70
Concentrating solar thermal power (total)	GW	0.7	1.3	1.8
Wind power capacity (total)	GW	159	198	238
Solar hot water/heat capacity (total)	GW_{th}	153	182	232
Ethanol production (annual)	billion liters	73.1	86.5	86.1
Biodiesel production (annual)	billion liters	17.8	18.5	21.4
Countries with policy targets	#	89	109	118
States/provinces/countries w/ biofuels mandates	#	57	71	72

Solar energy

Radiation from the sun is by far the most abundant energy source available on Earth. Only a small fraction of the energy sun provides Earth is utilized, the vast majority is released back from the surface of the planet (Barlev *et al.*, 2011). Moreover, the energy provided by most other renewable energy sources (e.g. hydroelectric power, wind power and energy from biomass) is indirectly derived from the sun.

The different techniques of producing energy from the sunlight can be divided into two main catagories. Firstly, solar photovoltaic (PV) uses solar cells to generate electricity directly through the photoelectric effect. Solar cell installation is fast growing, both regarding industrialization and research. Secondly, concentrated solar power (CSP) is a more indirect way of converting the sun radiation into electricity. CSP is a concept for different methods which are used to capture the thermal energy of the sunlight in power-producing heat processes (Barlev *et al.*, 2011; Hagfeldt, 2012).

During the period from 2006 to 2011, PV and CSP grew fastest of all renewable energy technologies, with an average annual operating capacity increase of 58% and 37%, respectively. In the EU, solar PV accounted for nearly 47% of all total electric capacity additions in 2011 (REN21, 2012). Worldwide, over 101 GW of electricity was produced by the solar PV technique in 2012 (EPIA, 2013).

Hydroelectric power

To date, hydroelectric power is the most utilized renewable energy source, providing nearly 1000 GW of electricity globally in 2011. The possibilities of growth for hydroelectric power is mainly restricted to the developing world, since a high proportion of the best territories for hydroelectric power plants are already utilized in most developed countries (Whittington, 2002; REN21, 2012).

Hydroelectric power plants can be divided into several groups. The most common type is called "high head", where water is stored behind a dam where it has high potential energy. The water is delivered through a large pipe to drive a water turbine and a generator. The amount of energy produced is controlled by the flow rate through the pipe and is set according to the power demand at each time. "Low head" hydroelectric power plants use only

a few meters head heights to the natural flow rate of the river. These have no capacity for storing water, which means that the seasonal flow rate of the river controls the amounts of energy produced. The third type is called "pumped storage", which is a high head plant with the additional option of using excess generation capacity to pump water from lower to higher reservoirs when the electricity demand is very low (Whittington, 2002). The other basic types of hydroelectric power plants are tide power and underground power stations.

Globally, approximately 25 GW of new hydroelectric capacity was installed in 2011, which is an increase of nearly 2.7% from 2010. The most active region in building new hydroelectric power plant was Asia, whereas many developed countries focused more on retrofits of existing facility in order to improve the output and efficiency. Around 71% of the electricity produced from renewable energy sources in 2011 was from hydroelectric power plants, 970 GW (REN21, 2012).

Wind power

Hydroelectric power and wind power are both renewable energy sources, with a common feature of being indirectly driven by the sunlight. The sun both increases the potential energy of water through elevation by evaporation, and also accounts for wind moving across the surface of the Earth by heating the air, which leads to different air pressure between geographical areas. In the 1980's, the utilization of wind for electricity production became one of the most important renewable energy sources in some developed countries. At the end of the decade, a total number of 16,000 wind turbines provided 1% of the electricity in the state of California. Moreover, 3% of all the electricity in Demark was produced by wind power plants (Gipe, 1993). The worldwide electricity production by wind power in 2011 was 238 GW, which was an increase of approximately 20% from the prior year. China was the largest wind power producer, accounting for nearly 44% of the global market (REN21, 2012).

The most common wind turbine is a large tower with three blade rotor, a hub and a nacelle. The rotor faces into the wind and rotates in a vertical plane. The location of wind turbines is very important since the resource available depends strongly on the on the annual mean wind speed. Many of

the most suitable territories for wind power production in Europe are in the northern part of England, in Scotland and on the north-west coast of Ireland. The cons of these areas are mostly concerning a long distance from the highest populated areas in the United Kingdom and Ireland (Leithead, 2007).

Geothermal energy

Geothermal energy is another renewable energy source available where heat energy from deep in the Earth is brought to the surface. The use of hot fluids, reached by drilling into reservoirs, depends on the temperature and the pressure of the fluids. Electricity can be generated from high temperature geothermal areas (over 149°C), whereas low temperature areas (under 149°C) are utilized for house heating and industrial processes. The origin of the geothermal fluids is rainwater, which penetrates deep into the Earth's crust and is heated by magma bodies, which are either still in a fluid state or undergoing solidification. In other areas the heat accumulation is due to particular geological conditions of the crust, where the heat gradient moving from the Earth’s interior towards the surface has anomalously high values. Impermeable rocks prevent the fluids from reaching up to the surface and keep them under high pressure at hot temperatures (Barbier, 2002; Whittington, 2002).

Since the conventional utilization of geothermal energy is quite limited to its location and ultimate potential for supplying electricity, a different technique of using geothermal energy is developing and showing promising advantages for geothermal energy production in the future. These are called enhanced geothermal systems (EGS) and are possible over a much greater geographical range. Heat-mining technology is used to produce a closed loop; cold water is pumped deep into the ground where it gets extremely hot and is used to produce electricity when it reaches the Earth's surface from another borehole (Tester *et al.*, 2007).

According to the Geothermal Energy Association, the United States is the world's leading country in geothermal energy production, with an installed geothermal capacity of 3.4 GW in February 2013 (GEA, 2013). The estimated global geothermal electricity was around 11.2 GW in 2011. Totally 0.14 GW of geothermal electricity generating capacity was added in 2011 in

the three leading countries in new geothermal power plants that year; the United States, Nicaragua and Iceland (REN21, 2012).

Bioenergy from biomass

Biomass became the first renewable energy source for humans when human ancestors learned to use fire and burn biomass. The term biomass covers all the complex chemical compounds produced by plants and microorganisms through the process of photosynthesis. When biomass is burned, the products are mainly carbon dioxide and water. At the same time, the chemical energy bound in the molecules present in the biomass are converted to kinetic energy (BER, 2009).

In many developing countries, biomass has a very important role as a "fuelwood", such as for cooking and house heating. This use of biomass, called "traditional", has a very low energy efficiency. Although the energy source is renewable the use of it is in most cases far from being sustainable, since the consumed biomass is not actively replaced with new plants and the utilization is leading to severe deforestation (Goldemberg & Coelho, 2004). In India, for instance, over 700 million people live in homes where biomass-based energy sources are used for cooking (Kurchania et al., 2010). On the other hand, the sustainable production of solid biomass as an energy source is growing as well (REN21, 2012).

2.3 Biofuels

In contrast to the "traditional" use of biomass, the so-called "modern" use includes electricity generation, heat production and production of biofuels, even from agricultural and forest residues and solid waste. This utilization of biomass is both considered renewable and sustainable (Goldemberg & Coelho, 2004).

Even though solid bio-char is defined as a biofuel, the term is mostly used to describe different types of renewable energy sources produced from natural materials, which are either in a liquid or gaseous state at room temperature. Biodiesel, methanol, ethanol, butanol, hydrogen and methane are the most common biofuels produced. These energy sources have many important advantages which make them a promising future energy source.

The benefits of biofuels are for instance economical, environmental and energy security related. Production of biofuels is far less dependent on natural resources compared to the fossil fuel industry. This gives countries that need to import most of their energy for industry and transport an opportunity to reduce their expenses on essential energy dramatically (Demirbas, 2009).

Biomethane

Methane-rich biogas (biomethane) is a versatile renewable energy source which can be used for power and heat production, as well as gaseous vehicle fuel. Wastes, residues and energy crops can be used locally to produce biomethane. Its production through anaerobic digestion has been evaluated as one of the most energy-efficient and environmentally beneficial technology of bioenergy manufacture (Weiland, 2010).

The biogas produced through anaerobic digestion is a mixture of methane (CH_4) and carbon dioxide (CO_2) in the most common proportions of approximately 65% and 35%, respectively. Biogas combustion releases carbon dioxide and water but no harmful gas compounds (Kurchania *et al.*, 2010).

The large-scale production of biomethane is expanding in some parts of the world, especially in Europe. Germany is leading the region's output. In 2011, nearly 61% of the region's biogas was produced in Germany, where the industry's average annual growth in the first decade of the century was 18%. Europe's biogas production increased over 31% in 2010, to 460 PJ (REN21, 2012).

Biodiesel

Biodiesel is a synthetic diesel-like fuel which can be produced in various ways and either replace diesel fuels (which may require some engine modifications) or blended with petroleum diesel. It is mostly produced from vegetable oils, animal fats or waste cooking oil (Demirbas, 2009). Biodiesel consists of methyl esters of fatty acids, produced by the transesterification of triacylglycerides (Saka & Kusdiana, 2001).

Studies have shown that the use of biodiesel fuels derived from vegetable oils on normal diesel engines can reduce the release of greenhouse gases and dangerous chemicals significantly. Ilkilic and Yucesu (2008) presented results from their experiments with cottonseed oil on a diesel engine, stating that the performance of the biodisel was comparable to normal diesel fuel. A reduction of 30% in CO and 25% in NO_x emissions were observed (Ilkilic & Yucesu, 2008). Moreover, biodiesel has benefits in comparison to petroleum diesel in terms of sulfur content, flash point, aromatic content and biodegradability (Saka & Kusdiana, 2001).

In 2011, approximately 11 billion liters of biodiesel were produced in 190 biodiesel plants in the United States. Germany and Spain are the leading biodiesel-producing countries in Europe with 22% and 20% of the continent's output in 2011, respectively. That year, the total biodiesel production in Europe was 25.1 billion liters (REN21, 2012).

Bioethanol

Human have used ethanol for a long time. Neolithic people consumed ethanol 9000 years ago, according to research on clay jars recovered from China which were made and used by this human ancestor (Service, 2013). Although ethanol is best known for human consumption, ethanol has gained a new purpose and is now becoming one of the most interesting options of future energy sources.

Ethanol was initially used as a fuel for engines in Germany in the nineteenth century (Nardon & Aten, 2008). Other types of alcohols were used as well, but among them ethanol is known as the most suited renewable, bio-based and eco-friendly fuel for spark-ignition engines (Demirbas, 2009). In the dawning days of the car history, Henry Ford became first to introduce ethanol as a car fuel. The Ford Motor Company's Model T from 1908 could be powered by ethanol. The importance of ethanol as a fuel remained low for most of the 20th century since it was subordinated by petroleum fuels. On the other hand, raising petroleum oil prices, costs of oil importing and the issues of renewability and sustainability has increased the interest in using ethanol as a fuel in recent decades (Service, 2013).

In comparison to petroleum fuel, ethanol has several important benefits other than being both a renewable and sustainable energy source. Ethanol has

an octane number of 108, higher than petroleum fuels, which allows for a higher compression ratio before being subjected to pre-detonation. Moreover, theoretical efficiency advantages are achieved by a shorter burn time and leaner burn engine. On the other hand, a lower energy density than gasoline, lower flame luminosity, lower vapor pressure, higher miscibility with water, its corrosiveness and toxicity to ecosystems are the biggest disadvantages of ethanol as an engine fuel (Demirbas, 2009).

Pure ethanol can be used directly as a car fuel for cars with specifically designed engines. A more common use is though to blend anhydrous ethanol with gasoline. In the United States ethanol is blended 10% to gasoline, which does not require any engine modification for normal cars. Considerations of raising the ethanol proportion to 15% have received mixed opinions, since investigations are not absolutely unanimous about the effect of higher ethanol concentrations of vehicle engines (Demirbas, 2009; Service, 2013).

Two different methods are mainly used for ethanol production. The first one is to synthesize ethanol from ethylene, derived from hydrocarbon. This method is preferred for industrial use where the purity of ethanol is extremely important. The second method is to ferment biomass with the use of microorganisms, and the product is generally called bioethanol (Cadenas & Cabezudo, 1998).

The large-scale production of bioethanol as a fuel began in Brazil in the 1930's, with sugar extracted from sugar cane was used as the substrate. After World War II the bioethanol demand decreased as a result of low petroleum oil prices, but rose again in the oil crisis of the mid-70's. Ever since the oil crisis, there has been a continuous increase in bioethanol production in the United States (Sveinsdottir *et al.*, 2011).

The United States Congress passed the Clean Air Act Amendments in 1990, which required the use of gasoline addictives (oxygenates) in order to increase the oxygen content of gasoline and make engines burn more cleanly. The most commonly used gasoline addictive was primarily methyl *tert*-butyl ether, MTBE. Health complaints in regions where MTBE was used lead to thorough investigations of the effects of the oxygenating compound, including both epidemiological investigations of large human populations as well as laboratory studies using animal models. California and New York banned the use of MTBE, which lead to a nation-wide ban in 2004 (Service, 2013; Phillips *et al.*, 2008).

The United States Congress established the Renewable Fuel Standard law (RFS) in 2005, followed by an update in 2007. This is a very important step to guarantee a market for renewable fuels as it mandates that fuels incorporate increasing amounts of renewable components into gasoline. Figure 3 shows the estimated increase in ethanol production according to RFS through the year 2022, when the amount of ethanol production in the United States is predicted to reach 136.3 billion liters (Service, 2013). This means that the ethanol production is expected to increase almost 27-fold in the first 22 years of the century, but the production was 5.1 billion liters in 2000. In 2011, 52.6 billion liters of ethanol were produced in 209 biorefineries located in 29 states (RFA, 2012). Even though two new plants were installed in 2012, the annual production decreased to 50.3 billion liters - making it the first year of recession since 1996. The main reasons were claimed to be the worst drought in the United States in decades, record prices for corn, and a drop in demand for gasoline due to economic reasons (RFA, 2013).

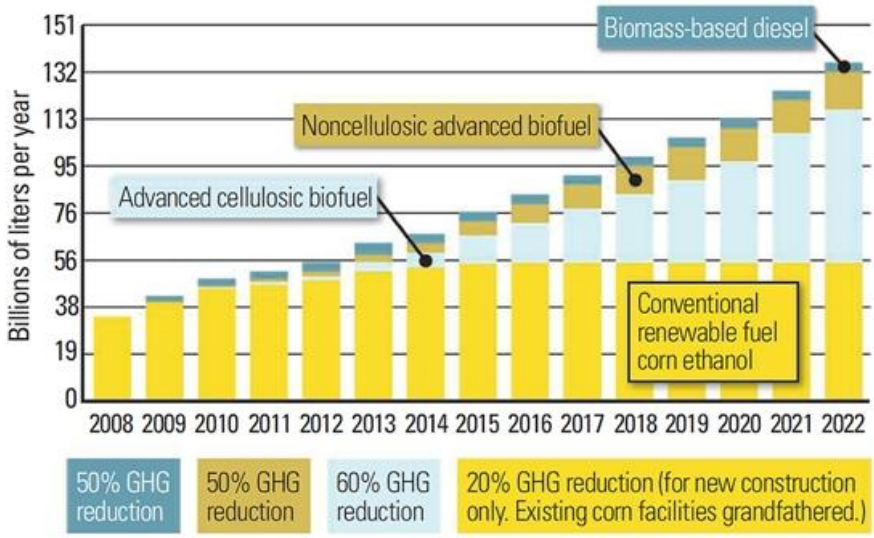

Figure 3. Estimated increase in biofuel production in the United States according to the Renewable Fuel Standard (RFS) law.

Together, United States and Brazil accounted for approximately 89% of the global ethanol production in 2010 (Sveinsdottir *et al.*, 2011). In 2011, China was the third largest bioethanol producer, producing 2.1 billion liters. It was followed by Cananda, France and Germany, producing 1.8, 1.1. and 0.8 billion liters, respectively (REN21, 2012).

Although there is still a long road ahead making bioethanol account for a large proportion of the fuel market, the progress of the last decade is very promising. Studies have shown that the use of bioethanol as a fuel reduces greenhouse gas emissions by 30-49% compared to gasoline, so there is no doubt about the fact that further substitution of fossil fuels by bioethanol will help reaching the goal of making the global energy utilization sustainable (RFA, 2012).

Biohydrogen

Hydrogen, the most abundant element in the universe, has been an industrially important compound for a long time. It is for instance used for the metal-catalyzed hydrogenation of many products (e.g. heavy oils in gasoline production, foods and ammonia as fertilizer) and as en electron donor for reducing numerous water pollutants. In the United States, approximately 10^8 m^3 of hydrogen are sold annually (Lee *et al.*, 2010).

In contrast to other common fuels, hydrogen is not chemically bound to carbon, which means that the only products of hydrogen combustion are water vapor and heat energy. Therefore, the utilization of hydrogen as fuel is considered very environmental-friendly. Hydrogen has the highest energy content per weight of any known fuel. (Nath & Das, 2004).

Hydrogen can be produced in several different ways. Most of the hydrogen gas production today is generated from fossil fuels through thermo-chemical processes. Some of the most common are hydrocarbon reforming, coal gasification and partial oxidation of heavier hydrocarbons. These methods are neither environmental-friendly nor using renewable feedstocks (Show *et al.*, 2011). Another possibility for hydrogen production is electrolysis of water. The method became the first commercial technology to produce hydrogen in the 1920's, but was outrun by fossil fuel methods in the 1960's. Even though electrolysis of water is more suitable than hydrogen

production from fossil fuels in terms of renewability and sustainability, the need for electricity in large quantities and water are two of the main drawbacks (IEA, 2006). Research on electrolysis of seawater for hydrogen production is gaining interest, since the advantages of using the abundant saline water resources are obvious. Some technical development for the method is though still needed (Abdel-Aal *et al.*, 2010). Among other possibilities of hydrogen production are the use of wood for pyrolysis technology, the use of natural gas and reformation of alcohols (IEA, 2006).

The most promising methods for hydrogen production in the future are by biological processes. They are less energy-intensive than chemical and electrochemical processes, since they are most often carried out at ambient temperatures and pressures. Bacteria and algae can be used to produce biohydrogen by several different methods; direct and indirect biophotolysis, photofermentation and dark fermentation. Light-driven decomposition of water in the presence of green algae is called direct biophotolysis. If cyanobacteria is used instead of algae, the process is called indirect biophotolysis (Nath & Das, 2004). The difference between photofermentation and dark fermentation is that the photosynthetic bacteria used for photofermentation requires light for the biological process, whereas the fermentative bacteria used for dark fermentation does not need light (Keskin *et al.*, 2011). Each of these different methods has it's pros and cons, but they are all still at investigation levels and not yet used for large-scale production (Keskin *et al.*, 2011; Show *et al.*, 2011). The process of dark fermentation will be described thoroughly in Chapter 2.6.

2.4 Biomass

As mentioned earlier, biomass refers mainly to all the complex chemical compounds produced by plants and microorganisms though the process of photosynthesis. The chemical bonds of these compounds contain potential energy which can be utilized as energy source (BER, 2009). Biomass is the only renewable primary energy source that is suitable for production of alternative transportation fuels such as bioethanol and biodiesel in the short term (Alvira *et al.*, 2010).

In 2006, biomass contributed about 46 EJ/year of energy in the form of solid, liquid or gaseous fuels. Estimations regarding the development of energy derived from biomass in the year of 2050 are very diverse; vary from 30 EJ/year to 1000 EJ/year. This enormous difference between studies is explained by different assumptions regarding several different factors that influence the availability of biomass for energy production. Among these factors are the availability of land, achievable yield levels of crops and technical progress in bioenergy production (Erb *et al.*, 2012).

Biomass is a very promising feedstock for future energy production for several different reasons. It is a renewable source that could be utilized in a sustainable manner. The use of biomass derived fuels has many positive environmental aspects, including no net carbon dioxide releases, very low sulfur dioxide emissions, and low ash production compared to coal combustion. Several diverse biofuels can be produced from biomass and the resource is to a great extent domestic which results in an economical benefit. Moreover, the utilization of agricultural and forestry residues as well as municipal solid wastes offer enormous opportunities for future energy production (Demirbas, 2008; Saxena *et al.*, 2009).

Starch

Starch is a polysaccharide, produced by all green plants, which stores chemical energy converted from sun radiation by the process of photosynthesis. Two different forms of this polymer exist; amylose and amylopectin (Figure 4 and Figure 5), both consisting entirely of D-glucose monomers. Amylose has an unbranched, helical structure where the glucose monomers are joined by (α-1→4) linkages. Amylopectin, which consists of

D-glucose backbone composed of (α-1→4) glycosidic bonds, has a more complex structure since the polymer is branched with (α-1→6) linkages at the branch points. The synthesis of starch granules makes plants capable of stockpiling surplus glucose, which can be withdrawn from this carbohydrate "bank" by hydrolysis, which enzymatically cleaves the bonds between the glucose monomers (Campbell & Reece, 2005).

When starch is used as a feedstock for biofuel production, depolymerization by a process called hydrolysis is needed to cut down the polymer structure to monomer units. At industrial levels, this was traditionally done by treatment of acid, but has been replaced by the more efficient method of using enzymes known as amylases. Most commonly these enzymes are obtained from thermoresistant bacteria like *Bacillus licheniformis*, fungi, or from engineered strains of *E. coli* or *B. subtilis* (Sanchez & Cardona, 2008). The enzymatic hydrolysis of starch is mainly performed by α-amylase and β-amylase. The α-amylase starts the digestion by hydrolyzing the internal (α-1→4) linkages, producing short polysaccharide fragments or oligosaccharides. β-amylase follows by working

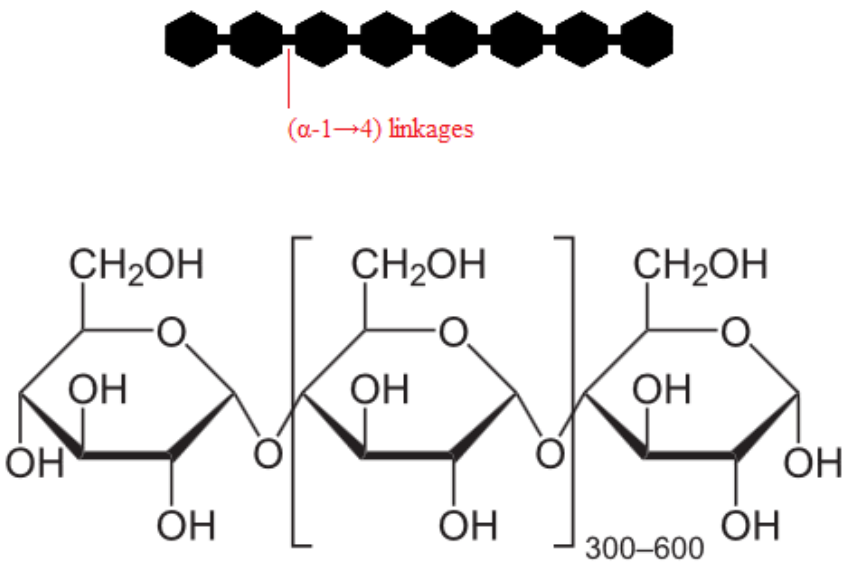

Figure 4. Structure of amylose molecule. Unbranched chain of glucose monomers, linked together with (α-1→4) bonds.

on the non-reducing ends of the oligosaccharides, cleaving off two glucuse units at a time, resulting in the disaccharide maltose (Nelson & Cox, 2008).

To date starch has been one of the most important feedstock for biofuel production, mostly derived from maize, wheat, potatoes, cassava, grains and tapioca (Le Corre *et al.*, 2010; Panagiotopoulos *et al.*, 2013). In the United States, maize has been the major biomass source of the bioethanol industry (Chen *et al.*, 2013).

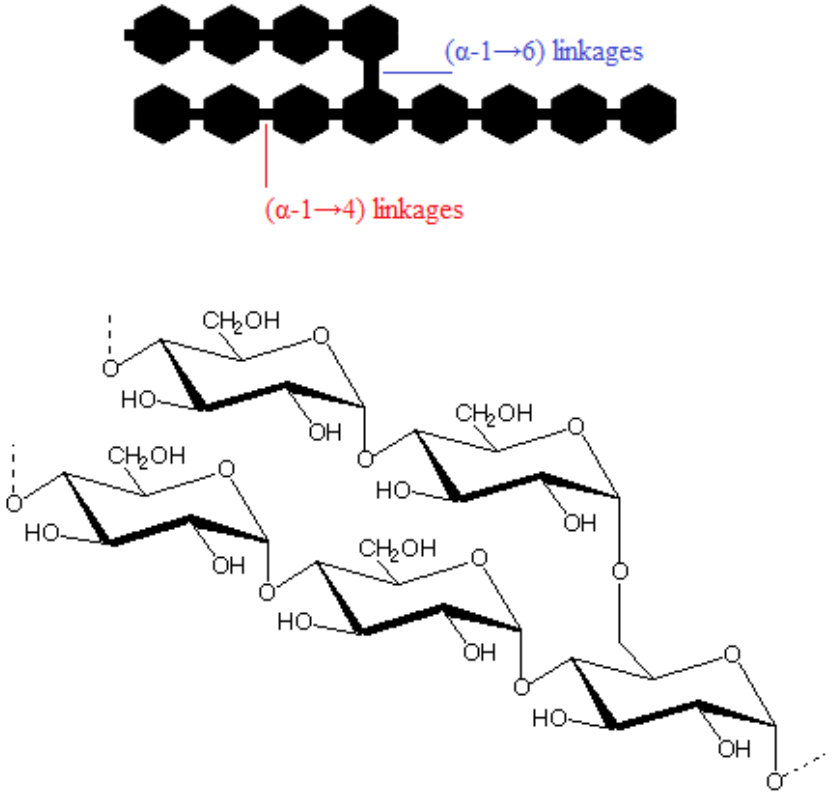

Figure 5. Structure of amylopectin molecule. Branched chain of glucose monomers. The monomers of each branch are linked together with (α-1→4) bonds, while the branch points have (α-1→6) linkages.

Lignocellulose

To date, the vast majority of biofuels production has been from starch and sugars and are called "first generation" biofuels. Relatively simple sugar polymers derived from crops such as corn, wheat and sugarcane have been used for this process, since the isolation of the sugars and conversion to biofuels by microorganisms both is rather easy and cost-effective. In context with increased biofuel output in recent years, first generation biofuel production has received strong criticism since it results in an undesirable direct competition with the food supply. The increased demand on feedstock has already lead to an increase in food prices. In light of the expected growth of the biofuel industry in the next years and decades, it is clear that the choice of raw material has to be thoroughly considered (Martin, 2010; Binod *et al.*, 2010).

The "food vs. fuel" debate is one of the most important factors leading to increased interest in second generation production of biofuels, where lignocellulose is the raw material instead of simple sugar monomers. Lignocellulose is a generic term for describing the main constituents in plants; it is a complex matrix comprising many different polysaccharides, aromatic heteropolymers and proteins. It includes for instance agricultural residues, dedicated energy crops, sawdust, wood chips, and municipal paper waste. Lignocellulosic biomass is cheap and abundantly available and is produced as a by-product in agricultural industry which is often defined as waste. It requires less agricultural land, watering and fertilizing than first generation production raw materials. Therefore it is a very promising renewable feedstock (Martin 2010; Binod *et al.*, 2010; Sarkar *et al.*, 2012). The complex structure of lignocellulosic biomass and difficulties in its separation and degradation of the different components are the main reasons why this type of biomass is not already a leading raw material in biofuel production. Increased knowledge about plant cell wall degradation and further technological progress, decreasing the high cost of lignocellulosic biomass utilization, is hoped to promote a revolution towards second generation production of biofuels in the future.

Plant cells are encapsulated within a complex wall (Figure 6), made out of several different components, and is crucial for the form and function of plants. The wall plays an important role in cell-to-cell adhesion, signaling, providing structural rigidity, defense against microbes and fungi, and

numerous growth and differentiation processes. Although the construction of all plant cell walls have some general things in common, it is clear that the composition between plant species or even between cells of the same individual plant can vary dramatically. The primary cell wall consists mainly of the polysaccharides cellulose, hemicellulose and pectin, as well as structural proteins and aromatic compounds. These structural components are bound together with hydrogen, covalent and ionic bonds. It is common that on a dry weight basis, approximately 30% of the primary wall is cellulose, 30% hemicellulose, 35% pectin and 1-5% structural proteins. Major deviations from these proportions are for instance observed by some grass types. Primary walls of growing maize coleoptiles consist of approximately

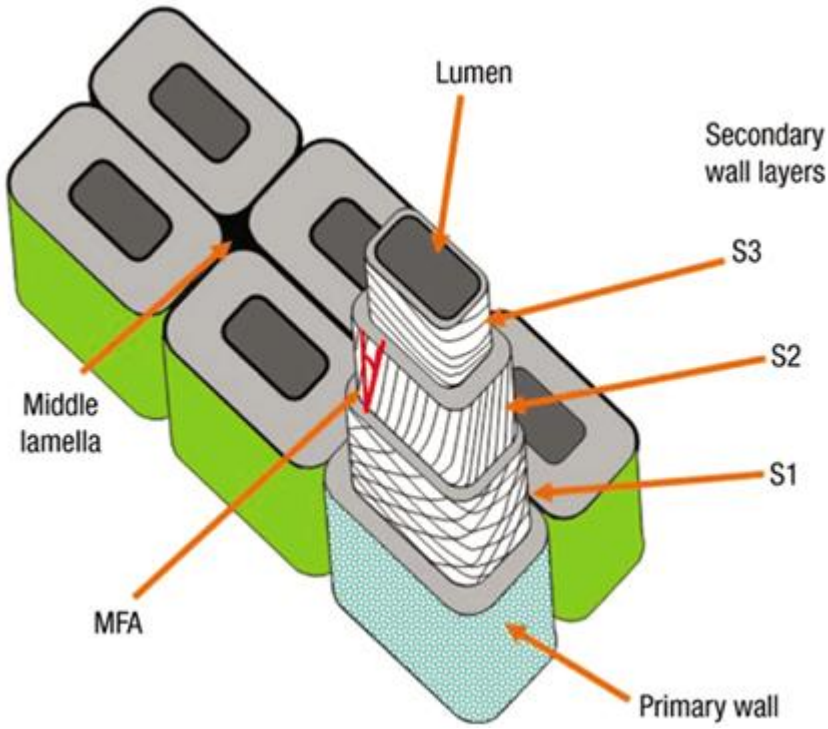

Figure 6. Schematic layered structure of plant cell walls. The secondary wall layers form between the lumen and the primary wall. The gray lines in the secondary wall layers represent idealized cellulose microfibrils. The cellulose microfibrils in the middle secondary layer appear twisted with respecto to the cell's vertical axis. The angle they form, called the microfibril angle (MFA), plays a crucial role in determining the stiffness of the wood (Kretschmann, 2003).

25% cellulose, 55% hemicellulose and 10% pectin (Cosgrove, 1997; Caffall, 2009).

In many plant cells, the cell wall is thickened and further strengthened by the addition of a secondary wall. Although different between plant cells, the secondary wall often becomes extremely thick in comparison to the primary wall. Secondary walls have higher proportions of cellulose than primary walls, and far less or even no pectin. The most obvious distinguishing feature of secondary walls are complex networks of aromatic compounds, called lignin (Caffall, 2009; Buchanan *et al.*, 2000).

Cell walls function long after the cells that produced them are dead and desiccated, which means that they account for the vast majority of lignocellulosic biomass (Buchanan *et al.*, 2000). As a result, the dry weight of lignocellulosic biomass typically consists of 36-61% cellulose and 13-39% hemicellulose. The rest is primarily lignin (Olsson & Hahn-Hagerdal, 1996).

Cellulose

Cellulose is the most abundant organic compound on Earth. It is estimated that plants produce almost 100 billion tons of cellulose each year (Campbell & Reece, 2005).

Like starch, the cellulose polymer is an unbranched chain of D-glucose monomers. The ring form of glucose has two different orientation possibilities for the hydroxyl and the hydrogen groups attached to the anomeric carbon (C_1), distinguishing between alpha (α) and beta (β) glucose (Figure 7). Cellulose consists of β-glucose monomers whereas starch contains α-glucose monomers, resulting in a difference in the glycosidic linkages between monomers of these two polysaccharides. This gives the two molecules distinct three dimensional shapes. Whereas a starch molecule is helical, a cellulose molecule is straight, never branched and its hydroxyl groups are free to make hydrogen bonds with other cellulose molecules lying parallel to it (Campbell & Reece, 2005).

(Figure 8) is a good overview of how cellulose is structured in cell walls. A dimer of two β-glucose monomers, cellobiose, appears in a repeated segment. Each cellulose chain forms hydrogen bonds with parallel chains, resulting in long paracrystalline assemblies called microfibrils. On average, each microfibril in plant cell walls is 36 individual cellulose chain thick. Each cellulose chain can consist of several thousand monomers, but

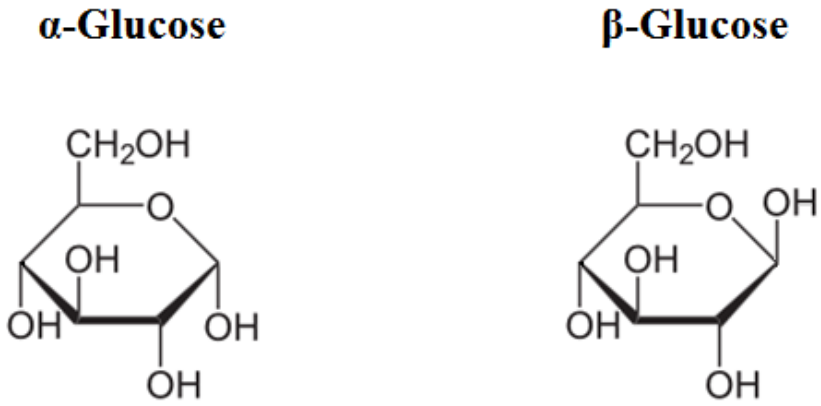

Figure 8. Structure of α-Glucose and β-Glucose. The only difference is the orientation of the hydroxyl group on carbon number 1.

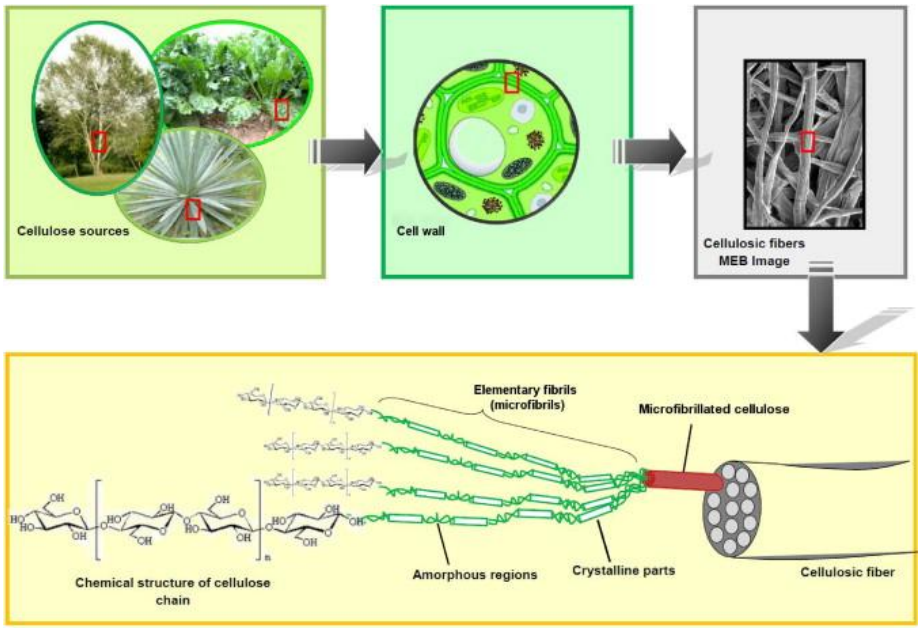

Figure 7. structure in plants, from sources to molecules (Lavoine *et al*., 2012).

individual chains begin and end at different places within the microfibril, which makes each microfibril able to contain thousands of individual cellulose chains and reaching lengths of hundreds of micrometers (Buchanan *et al.*, 2000; Lavoine *et al.*, 2012). Microfibrils form larger fibrils with assistance of other lignocellulosic components than cellulose, which will be discussed later.

Most organisms do not possess enzyme systems capable of degrading the β linkages of cellulose. The enzymes used to degrade starch cannot hydrolyze cellulose in light of the different linkages and the different structure of the polymers. A number of bacteria and fungi, however, produce cellulases, enzymes which hydrolyze the β-1,4 glycosidic bonds of cellulose. Even those organisms are generally not able to degrade crystalline cellulose completely, since the extensive intermolecular bonding pattern of cellulose generates a fascinating structure which is resistant to microbial degradation (Bayer *et al.*, 1998).

The main groups of cellulases are endoglucanases, exoglucanases and cellobiases. Organisms which have shown ability to degrade cellulose either have a collection of different free cellulytic enzymes or possess multicomponent complexes thereof, called cellulosomes. Recombinant bacteria strains are used to produce cellulases for industrial use (commonly strains of *E. coli* and *Z. mobilis*), but a deeper understanding of lignocellulosic substrate and enzyme systems is still needed for further progress in utilization of lignocellulosic biomass (Bayer *et al.*, 1998; Jung *et al.*, 2012). Cellulose hydrolysis will be discussed further in chapter 2.5.

Hemicellulose

The second of the three main components of lignocellulosic biomass is a group of cross-linking glycans called hemicellulose. Hemicellulose constitute complex branched heteropolysaccharides, which are mainly divided to four groups; xylans, mannans, mixed linkage β-glucans and xyloglucans. These heteropolymers contain mostly L-arabinose, D-galactose, D-glucose, D-mannose and D-xylose. The most common acids present in hemicellulosic biomass are D-glucoronic acid, D-4-*O*-methylgalacturonic acid and D-galacturonic acid. Methyl groups and acetyl groups are attached to the carbon chain to various degrees. The composition of hemicellulose varies greatly

with the origin of the hemicellulosic material (Buchanan *et al.*, 2000; Ebringerova *et al.*, 2005; Olsson & Hahn-Hagerdal, 1996; Girio *et al.*, 2010).

Hemicelluloses can form hydrogen bonds to cellulose microfibrils. Even though these polymers occur in much shorter molecular chains than cellulose, they are long enough to span the distance between different microfibrils and link them together to form a tight network (Buchanan *et al.*, 2000; Demirbas, 2008).

The most abundant polymer of hemicellulose is xylan, formed by (β-1→4) linked D-xylopyranoside monomer units (Figure 9). In hardwood species, xylan generally accounts for over half of the hemicellulose dry weight, but somewhat less in softwood. The complex structure of xylan requires several different enzymes for complete hydrolysis. Most important are endo- and exo-acting β-1,4-xylanases and β-xylosidases, which dismantle the xylan backbone into xylose units (endo-acting enzymes can cleave a polymer at any location, whereas exo-acting enzymes can only accept the polymer via its termus). Among other enzymes needed to cut off side groups and other substituents are α-L-arabinofuranosidases, α-glucuronidases, acetylxylan esterases, ferulic acid esterases and *p*-coumaric acid esterases (Girio *et al.*, 2010; Deutschmann & Dekker, 2012).

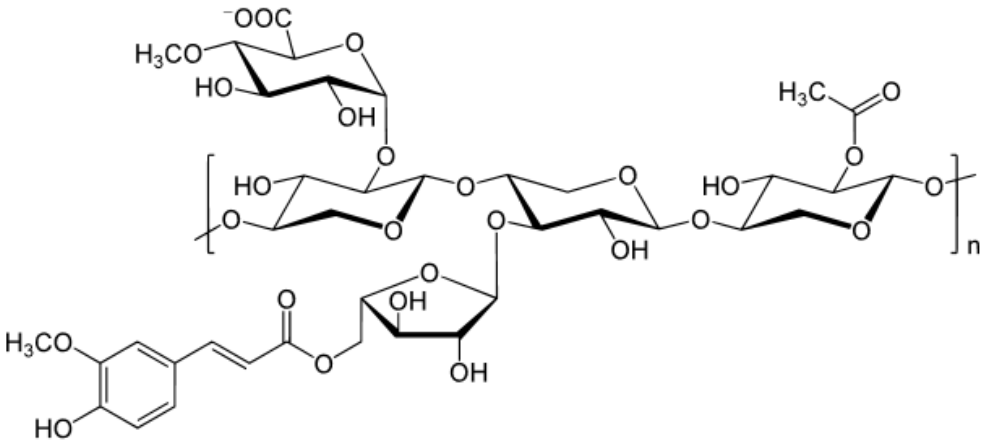

Figure 9. The structure of xylan. The three monomers appearing in the brackets are repeated and form the backbone of the molecule.

Lignin

The third and last main component of lignocellulosic biomass is lignin. It is the most distinguishing feature of secondary plant cell walls, but with a few exceptions it is not found in primary walls (Buchanan *et al.*, 2000). Lignins are random heteropolymers of aromatic compounds which have the biological roles in plants to provide structural strength by binding tightly to cellulose and hemicellulose, provide sealing of the water conducting system that links roots with leaves and protects plants for bacterial and fungous digestion (Service, 2013; Demirbas, 2008; Campbell & Reece, 2005).

There are three main units in the lignin polymer; *p*-hydroxyphenyl (H), guaiacyl (G) and syringil (S). They are derived from three different monolignols which differ in the number of methoxyl substituents on the aromatic ring; *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, respectively (Figure 10). The abundance and combinations of these main units vary between plant species, tissues, cell types and developmental

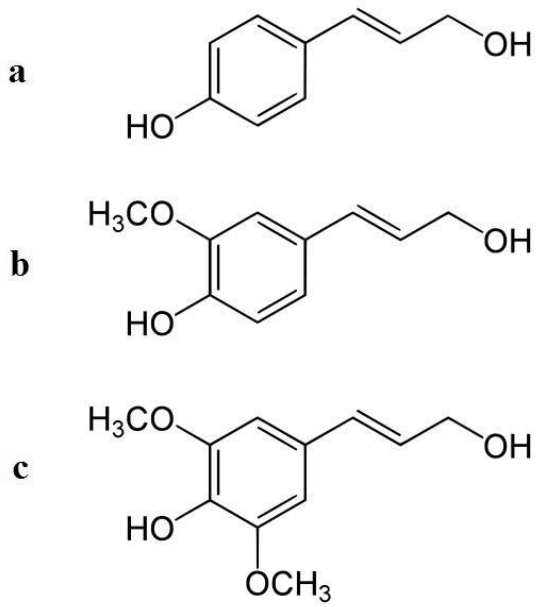

Figure 10. The monolignols generating the three main units of the lignin polymer. p-coumaryl alcohol (a) form H-units, coniferyl alcohol (b) form G-units and synapyl alcohol (c) form S-units (Vanholme et al., 2012).

stages. For instance, H units generally account for up to 5% of lignin in monocots, whereas only traces of H units are found in dicots (Vanholme *et al.*, 2012).

In contrast with cellulose and hemicellulose, lignin cannot be used for biofuel production (Cadenas & Cabezudo, 1998). It's extremely tight and complex structure, which immobilizes cellulases and blocks them from reaching the polysaccharides, is the major cause of lignocellulosic biomass recalcitrance to efficient industrial processing (Vanholme *et al.*, 2012). Interestingly, some bacteria and fungi produce enzymes which cut down the lignin structure to some extent, and thereby improving the access to the polysaccharides present in the biomass. This group of lignin modifying enzymes include different types of peroxidases and phenol-oxidases (laccases). Unfortunately, these enzymes have not shown to be practical for industrial-scale removing of lignin because of low rate of oxidation (Zhang *et al.*, 2012). Since plants can tolerate large variations in lignin composition, often without any effect on the plant, substitutions of some of the fractions of the traditional monolignols by alternative monolignols through genetic engineering might be an interesting way to tailor lignin in lignocellulosic biomass (Vanholme *et al.*, 2012). Technological progress and a better knowledge of how to isolate and degrade lignin in lignocellulosic biomass is the most important challenge in order to make lignocellulosic biomass a future energy source which can fulfill our need of energy in a sustainable way.

2.5 Pretreatment of lignocellulosic biomass

As discussed in chapter 2.4, the structure of lignocellulosic biomass is very complex and plants possess a strong defence mechanism to protect themselves from microbial degradation and environmental stress. It is extremely rare that microbes are able to utilize pure untreated lignocellulosic biomass as it appears in nature. One exception is the gram-positive thermophile *Anaerocellum thermophilum* which has been observed to degrade both untreated switchgrass and poplar (Yang *et al.*, 2009).

Generally, biomass needs to be pretreated in order to break down the lignin structure and disrupt the crystalline structure of cellulose for enhancing enzymes accessibility to the polysaccharides during hydrolysis. Research on pretreatment methods for lignocellulosic biomass focuses on identifying, evaluating, developing and demonstrating promising approaches that support the subsequent enzymatic hydrolysis of the treated biomass with lower enzyme dosages and shorter bioconversion time. Progress in this investigation field is one of the most important regarding biofuel production in the future. Pretreatment methods have been described as the second most expensive unit cost in the conversion of lignocellulosic biomass to bioethanol based on enzymatic hydrolysis. Feedstock cost is the only part that is more expensive (Alvira *et al.*, 2010).

Numerous different factors regarding pretreatment methods are important and have an impact on the effectiveness of the method (Alvira *et al.*, 2010; Yang & Wyman, 2008). When pretreatment methods are assessed, the following is desired:

- High yields for multiple crops, site ages and harvesting times
- Minimum heat and power requirements
- Biomass size reduction not required
- Operation in reasonable size and moderate cost reactors
- Highly digestible pretreated solid
- Obtaining high sugar concentration
- No significant sugar degradation
- Minimum amount of toxic compounds
- No solid waste residues produced
- Effectiveness at low moisture content
- High lignin recovery

A large number of different pretreatment methods have been described. Each method has it's advantages and disadvantages referring to the factors listed above. It is also important to keep in mind that the most suitable pretreatment method might vary between biomass crops, depending on the composition of each type of biomass.

In this chapter, pretreatment methods which belong to the following four groups will be discussed:

- Physical pretreatment.
- Chemical pretreatment.
- Physio-chemical pretreatment.
- Biological pretreatment.

Furthermore, hydrolysis (which is necessary to degrade large polysaccharides to small and simple sugars for fermentation) and the inhibition effects of pretreatment will be discussed in the last two subchapters.

Physical pretreatment

Physical pretreatment methods are based on the principle to reduce the particle size of the biomass by mechanical action. This increases the surface area of the biomass and reduces both the degree of polymerization and the crystallinity of cellulose. Physical pretreatment is often combined with other pretreatment methods, since most of them are much more effective if the particle size is reduced (Sousa *et al.*, 2009).

Mechanical comminution is a very common physical pretreatment method and often performed by a combination of chipping, grinding and milling the biomass (Alvira *et al.*, 2010). Grinding and milling can reduce the particle size of biomass at least to 0.2 mm, but it has shown that further particle size reduction below 0.4 mm has little effect on the yields of biomass hydrolysis (Agbor *et al.*, 2011).

The most important disadvantage of mechanical comminution is that the process requires high energy input. The energy required depends partially on the type of biomass used and the final particle size. Hardwood requires more energy than softwoods and more energy is needed to achieve smaller particle size. Even though studies have shown that milling increases biofuel yields produced from lignocellulosic biomass, this method is not likely to be

very economically profitable due to the high energy demand (Agbor *et al.*, 2011).

Extrusion is another possibility of a physical pretreatment. The materials are subjected by heating, mixing and shearing, which results in physical and chemical modifications during the passage through the extruder (Alvira *et al.*, 2010). Furthermore, the use of gamma rays can cleave (β-1→4) glycosidic bonds, resulting in a lower crystallinity. Apart from environmental and safety concerns regarding the use of gamma rays, both of the latter methods require very high amounts of energy (Agbor *et al.*, 2011).

Chemical pretreatment

Acid pretreatment methods

Chemical pretreatment methods are widely used in order to disrupt the structure of lignocellulosic biomass and increase the access of enzymes performing hydrolysis on the polysaccharides. Several different options could be defined as acid pretreatment methods, such as concentrated acid, dilute acid, steam explosion and liquid hot water pretreatment. Although steam explosion and liquid hot water pretreatment operate in acidic environment, these methods are in fact physio-chemical in will be described later (Sousa *et al.*, 2009).

The main effect of acid pretreatment methods is solubilizing a great part of hemicellulose and lignin from the plant cell wall structure, hydrolyzing hemicellulose and improving the enzyme accessibility to cellulose. Moreover, acid pretreatment hydrolyzes a part of the cellulose. The amount of cellulose hydrolyzed depends strongly on how the method is performed and increases with higher concentration of acid (Sousa *et al.*, 2009; Agbor *et al.*, 2011).

Among acid pretreatment methods, the direct addition of acid to the hydrolysate is most common. Sulfuric acid is the most common acid for pretreatment of biomass, but hydrochloric acid, phosphoric acid, nitric acid, trifluoroacetic acid and organic acids such as fumaric and maleic acids have been used for this purpose as well. Pretreatment with concentrated acid levels is not considered very suitable, although it is more effective in degrading sugars than dilute acid methods. The main reasons are equipment corrosion problems, the formation of inhibiting compounds such as aldehydes, acid

recovery and neutralization, and operational and maintenance costs. Diluted acid pretreatments, which generally include acid concentrations between 0.05% and 5%, are much more favorable and have been studied for pretreating wide range of lignocellulosic biomass. They are usually performed at temperatures ranging from 160°C to 220°C, but positive results have also been reported for temperatures as low as 120°C. This pretreatment method has several advantages. It is described as a very effective way to disrupt the structure of lignocellulosic biomass. No hemicellulose hydrolytic enzymes are needed to be added during enzymatic hydrolysis, since the acid takes care of the hydrolyzation of hemicellulose. Moreover, the formation of inhibiting products is not considered very high, compared to pretreatment with concentrated acids (Agbor *et al.*, 2011; Alvira *et al.*, 2010; Brodeur *et al.*, 2011). On the other hand, lignin remains on the surface of crystalline cellulose when this method is applied and has shown to block the hydrolyzing enzymes accessibility to the substrate to some extent, and the acids needed are relatively expensive (Agbor *et al.*, 2011; Li *et al.*, 2010).

Pretreatment with acid requires the use of an alkali to neutralize the hydrolysate after the treatment has been carried out (Agbor *et al.*, 2011).

Alkaline pretreatment methods

Pretreatment with alkali is another common pretreatment method. It involves the use of bases such as sodium hydroxide, potassium hydroxide, calcium hydroxide and ammonium hydroxide. Alkaline pretreatment methods cause degradation of ester and glycosidic side chains, resulting in a structural alternation of lignin, cellulose swelling, partial decrystallization of cellulose and partial solvation of hemicellulose. This reduces the non-specific binding during enzymatic hydrolysis and disrupts the lignocellulosic structure, which makes the polysaccharides more accessible for the hydrolyzing enzymes (Brodeur *et al.*, 2011; Sousa *et al.*, 2009). Pretreatment with alkali is an especially powerful method in the separation of lignin and is therefore most effective on biomass with high lignin content, compared to most other methods (Yang & Wyman, 2008). Following alkali pretreatment, acidic solution is needed to neutralize the hydrolysate.

Sodium hydroxide has been used for alkali pretreatment for many years. This compound disrupts the lignin structure and increases the accessibility of hydrolases, xylanases and other enzymes used for biomass

hydrolysis to cellulose and hemiellulose (Brodeur *et al.*, 2011). In a study on wheat straw biomass, the optimum sodium hydroxide concentration for the pretreatment was 1,5% and the optimum temperature for the process 20°C. The low observed optimum temperature results in the important advantage of low energy costs (Sun *et al.*, 1995).

Another common alkaline pretreatment method is the use of calcium hydroxide, often referred to as "lime pretreatment". This method is interesting since calcium hydroxide is the least expensive per weight of the four alkali compounds mentioned. Although lime pretreatment has not been investigated as much as pretreatment by sodium hydroxide, studies have shown that the method is very effective. The calcium can be recovered from the aqueous reaction system, regenerating calcium hydroxide by the lime kiln technology. On the other hand, lime pretreatment has shown to act slower than most other alkaline pretreatments and is not as affective for hardwood as for herbaceous plants or agricultural residues (Kaar & Holtzapple, 2000; Brodeur *et al.*, 2011; Yang & Wyman, 2008).

A general advantage of alkaline pretreatment methods is that they produce low amounts of toxic compounds, conversely to acid pretreatments. On the other hand, one of the major disadvantages of alkaline pretreatment is its low efficiency in solubilizing hemicellulose (Alvira *et al.*, 2010). A better knowledge of the cell wall chemical and ultrastructural modifications is needed to provide enzyme mixtures which are more efficient in degrading both cellulose and hemicellulose following alkaline pretreatment (Sousa *et al.*, 2009).

Other chemical pretreatment methods

Among other chemical pretreatment methods that have been described are ionic liquid, organosolv, ozonolysis, green solvents and wet oxidation. The first three of these methods will be described briefly.

Ionic liquid pretreatment is a method that has gained increased interest recently. These are liquid solvents which contain chloride, acetate or other moderately basic anions which enable the dissolution of cellulose be disrupting its hydrogen bond network. Moreover, the ions are very effective in separating the lignin from other parts of the biomass. Studies have shown that ionic liquid pretreatment is very promising, and is also capable of improving significantly the efficiency of hydrolysis in a combination with

other pretreatment methods. This method has to be investigated further, especially in terms of the recovery and reuse of ionic liquids in pretreatment, since they are very expensive (Li *et al.*, 2010; Ungurean *et al.*, 2011).

Organosolv pretreatment is based on using organic solvents (e.g. methanol, ethanol, ethylene glycol, glycerol, tetrahydrofurfuryl alcohol, dimethylsulfoxide, ethers, ketones, phenols) to solubilize lignin and hemicellulose. This process can take place in moderate temperatures, even at room temperature, but more commonly high temperatures (185-210°C) are used. In some organosolv processes acid catalysts are added to increase the sugar yields. The most important advantages of the organosolv pretreatment are that the organic solvents are easily recovered by distillation and that it can isolate lignin as a solid material but carbohydrates as syrup. Some of the main drawbacks are that the pretreated solids need to be washed with organic solvents previous water washing in order to avoid the reprecipitation of dissolved lignin, high solvent cost, and the method must be performed in a highly controlled environment due to the volatility of organic solvents (Zhao *et al.*, 2009a).

Ozonolysis pretreatment is based on using ozone (O_3) to attack lignin in the biomass, releasing soluble compounds of less molecular weight. Ozone is highly reactive towards compounds containing conjugated double bonds; ozone readily oxidizes lignin due to its high double bond content. The two main pros of ozonolysis pretreatment are low inhibitory compound formation and that it can be performed at ambient temperature (Garcia-Cubero *et al.*, 2009).

Physio-chemical pretreatment

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

Steam explosion

One of the most commonly used pretreatment methods for lignocellulosic biomass that uses both physical and chemical techniques is steam explosion. It is performed by subjecting the biomass to high pressurized steam at high temperatures for a short time followed by a rapid depressure of the system (Brodeur *et al.*, 2011; Alvira *et al.*, 2010).

This method is able to generate full sugar recovery, requires low capital investments, has low environmental impacts and is considered to be highly efficient. On the other hand, a part of the hemicelluloses are degraded during the method and some toxic compound which affect enzymatic hydrolysis are generated (Brodeur *et al.*, 2011; Tomas-Pejo *et al.*, 2010).

Liquid hot water

The liquid hot water pretreatment method uses water at high temperatures and pressures, similar to steam explosion. The main difference is that for the liquid hot water method, more extreme pressure (over 5 MPa) is used in order to keep the water in the liquid state, and no rapid decompression is needed. Normally the temperatures range from 160°C to 240°C over a time period from a few minutes up to an hour. This process solubilizes the hemicellulose, separating it from the cellulose-rich solid fraction. The structure of the lignocellulosic biomass is efficiently disrupted, but the substrates need to be handled further with enzymes (Brodeur *et al.*, 2011; Sousa *et al.*, 2009).

The advantages of this process are for instance that there is no need for acid, neutralization or degradation additives and inhibitory products are not produced in overwhelming amounts. The amount of inhibitory components can be reduced further by controlling the acidity of the hydrolysate, which restricts the number of monomeric sugars produced from the hemicellulosic oligosaccharides. However, high energy input is needed - more than for steam explosion since the pressure needs to be significantly higher (Brodeur *et al.*, 2011; Mosier *et al.*, 2005).

Ammonia fiber explosion (AFEX)

Ammonia fiber explosion (AFEX) is another physio-chemical pretreatment method for lignocellulosic biomass where high pressure is used to disrupt the biomass structure. Instead of using water, like in the two aforementioned physio-chemical methods, AFEX utilizes liquid anhydrous ammonia at temperatures ranging from 60°C to 100°C and high pressure (1.7-2.1 MPa) for short periods of time (approximately 5 minutes). When the high pressure is released, it results in a rapid expansion of the ammonia gas that causes physical disruption of biomass fibers and partial decrystallization of cellulose. All parts of the treated biomass remain in a solid state (Alvira *et al.*, 2010; Teymouri *et al.*, 2005).

The AFEX pretreatment has shown to allow recovery of plant proteins in their native functional form. It might be an interesting option to reduce the cost of plant biomass hydrolysis by using transgenic plants which express and release hydrolyzing enzymes during pretreatment, preventing the need for adding hydrolyzing enzymes following the pretreatment. However, this needs further investigations (Teymouri *et al.*, 2004). Both cellulose and hemicellulose are well preserved in this method. The main cons of AFEX are decreased efficiency for raw materials with high lignin content and high cost of the large ammonia amounts required (Teymouri *et al.*, 2005; Tomas-Pejo *et al.*, 2010).

Wet oxidation

Wet oxidation is a pretreatment method where biomass is treated with oxygen at temperatures from 170°C to 200°C and pressure from 1.0 to 1.2 MPa for 10-15 minutes. This disrupts the lignocellulosic structure and results in a cellulose-rich solid fraction and a hemicellulose-rich liquid fraction (Schmidt & Thomsen, 1998; Alvira *et al.*, 2010).

Some of the advantages of wet oxidation pretreatment is that it can easily be performed in a continuous process which favors scale-up, does not require a detoxification step, and efficiently removes lignin. On the other hand, the expensive oxygen required is the most important disadvantage of the method (Schmidt & Thomsen, 1998; Alvira *et al.*, 2010).

Other physio-chemical pretreatment methods

Many other pretreatment methods which combine both physical and chemical processes have been described. Among them are ammonia recycle percolation (ARP), supercritical fluid pretreatment (SCF), microwave pretreatment, ultrasound pretreatment and CO_2 explosion. These methods have generally been less studied than the four physio-chemical methods described earlier. It is clear that many methods that to date have not been widely used are promising and further research and technological progress might make them important for future pretreatment of lignocellulosic biomass (Brodeur *et al.*, 2011; Alvira *et al.*, 2010).

Biological pretreatment

As mentioned earlier, some bacterial and fungal strains produce extracellular enzymes which can take part in degrading lignocellulosic biomass. This offers an opportunity of pretreatment in a biological way. White-rot fungi (e.g. *Phanerochaete chrysosporium*, *Phlebia radiata*, *Dichmitus squalens*, *Rigidosporus lignosus* and *Jungua separabilima*) seem to be most suitable since they are capable of degrading lignin as well as hydrolyze cellulose, but brown-rot and soft-rot fungi have also shown positive results. The most important enzymes produced are lignin peroxidases, polyphenol oxidases, maganesse-dependent peroxidases and laccsaes (Agbor *et al.*, 2011; Lee, 1997).

Biological pretreatment methods have some advantages in comparison to other methods, including mild reaction conditions, high product yields, few side reactions, very low energy demand and low reactor resistance to pressure and corrosion. The main reason why biological pretreatments are not used at industrial levels is because they are very slow (normally takes 10-14 days) and need a lot of space. Moreover, careful growth conditions are required and some of the carbohydrates might be consumed by the microorganisms (Agbor *et al.*, 2011; Lee, 1997).

One possibility to degrade lignocellulosic biomass is to use several different microbes in a co-culture, where the strains are able to secrete different enzymes which altogether are able to hydrolyze the sugar polymers. Recent study where a mixture of microbes, mainly bacteria, called XDC-2 was used showed positive results. On the other hand, this method might be slow and have some drawbacks concerning utilization of the resulting sugars by the pretreatment microbes (Guo *et al.*, 2010; Hui *et al.*, 2013).

Enzymatic hydrolysis

Many pretreatment methods do not only disrupt the structure of lignocellulosic biomass, but perform partial hydrolysis on the substrate by degrading the polysaccharides to smaller units as well. However, most pretreatment methods need to be followed by a more complete hydrolysis in order to produce simple sugars which can be utilized by microorganisms for biofuel production. This is generally performed by enzymes.

As mentioned in chapter 2.4, three groups of enzymes are needed to carry out the hydrolysis of cellulose. These groups are endocellulases, exocellulases and cellobiases (β-glucosidases). Endocellulases start the enzymatic hydrolysis by disrupting the noncovalent interactions in the amorphous structure of cellulose, which breaks down the crystalline structure of cellulose microfibrils and liberates individual polysaccharide chains. Exocellulases follow by progressively convert the long cellulose chains to cellodextrins, short glucose chains which are soluble and highly biodegradable. Finally, cellobiases convert the cellodextrins to individual glucose monomers (Figure 11). Commercial enzyme mixtures used to hydrolyze cellulose often consist of endo- and exocellulases derived from fungal strains such as *Trichoderma reesei*, supplemented by cellobiases to mitigate product inhibition of cellulases by cellodextrins. These enzyme mixtures often contain over 80 different proteins, which many of do not have an important impact on hydrolysis of lignocellulosic biomass. This has led to investigations seeking to produce synthetic enzyme mixtures *de novo*, in order to get rid of the superfluous proteins and increase the efficiency of cellulose hydrolysis (Zhang *et al.*, 2012).

In contrast to cellulose, hemicellulose is a polymer that consists of many different sugar monomers and the composition varies between plants and even between tissues of the same individual. This makes the hydrolysis of hemicellulose more complex than the hydrolysis of cellulose. A diverse combination of enzymes is required for the complete hydrolysis of hemicellulose. Endoxylanases, exoxylanases and β-xylosidases are responsible for hydrolyzing the backbone, forming xylose monomers. Among other important enzymes for hydrolysis of hemicellulose are α-arabinofuranosidases, α-glucoronisidases and acetylxylan esterases (Zhang *et al.*, 2012).

As mentioned earlier, some bacteria and fungi produce enzymes that are capable of breaking down the lignin polymer to some extent, which increases the access to cellulose and hemicellulose. These lignin modifying enzymes include different types of peroxidases and phenol-oxidases of laccase-type. More efficient ways to hydrolyze lignin are under investigation since lignin degradation is one of the greatest hurdles in the utilization of lignocellulosic biomass (Zhang *et al.*, 2012).

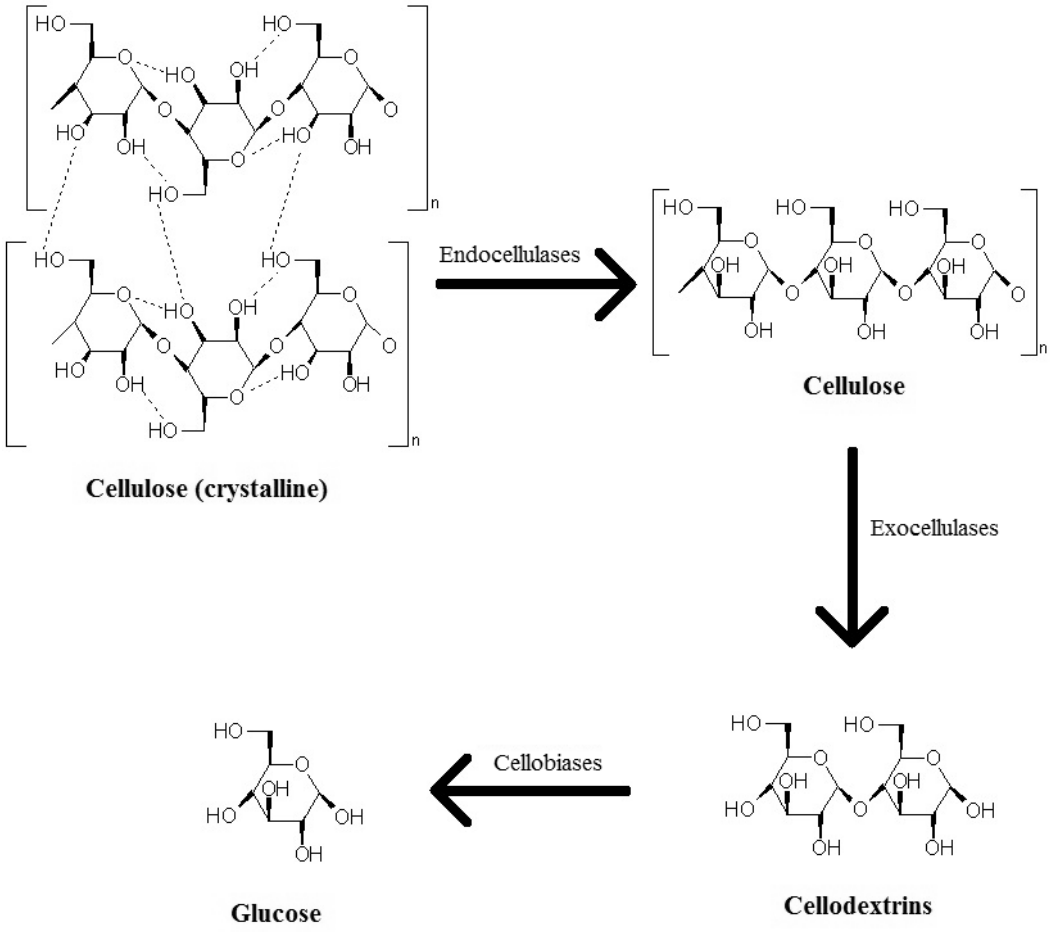

Figure 11. Hydrolysis of cellulose, from the crystalline structure to glucose monomers.

Investigations have shown that numerous different factors influence the rate and efficiency of enzymatic hydrolysis. Among them are cellulose cristallinity, cellulose degree of polymerization, substrate's available surface area, the lignin barrier, hemicellulose content, porosity and cell wall thickness. Many of this factors are affected by the pretreatment method chosen, which should be kept in mind in light of the importance of successful hydrolysis for the conversion of biomass to biofuels (Alvira *et al.*, 2010).

Inhibitory effect of biomass pretreatment

During the degradation of lignocellulosic biomass a broad range of components are released. For instance, a study showed that over 60 different compounds could be isolated from a steam-pretreated hydrolysate of birchwood and identified with a gas chromatograph. Some of these compounds can inhibit the growth of the fermenting organism used for biofuel production, even at very low concentrations. The effects of inhibitory compounds are also indirect since they cause increased environmental stress for the fermentative organism due to decreased water activity. Nature and the amounts of these compounds depend on different factors, such as the type of biomass used, the pretreatment method, the complete hydrolysis and the extent of recirculation in the process (Olsson & Hahn-Hagerdal, 1996; Palmquist & Hahn-Hagerdal, 2000).

These inhibitory compounds can be divided into three groups, based on their origin; weak acids, furan derivatives and phenolic compounds (Palmquist & Hahn-Hagerdal, 2000). The most common inhibitory compounds found in hydrolyzed lignocellulosic biomass are acetic acid, laevulinic acid, formic acid and lactic acid (weak acids); furfural and 5-hydroxymethylfurfural (furan derivatives) and vanillin, syringaldehyde, cinnamaldehyde, acetaldehyde and p-hydroxybenzaldehyde (phenolic compounds). Moreover, heavy metal ions such as chromium, copper and nickel can be present in biomass and have inhibitory effects (Stoutenburg *et al.*, 2011; Olsson & Hahn-Hagerdal, 1996).

Detoxification

Since inhibitory compounds can have a dramatic consequence on the efficiency of biofuel production, knowledge about inhibitors and how to

minimize their effect is very important (Olsson & Hahn-Hagerdal, 1996). The term detoxification is used for methods which have been employed in order to remove inhibiting compounds from hydrolysates prior to fermentation by microorganisms.

Detoxification methods are divided to three groups; biological, physical and chemical. Several strains of fungi have been used for biological detoxification. Peroxidase and laccase derived from the lignolytic fungus *Trametes versicolor* have for instance been used for an oxidative polymerization of low molecular weight phenolic compounds. Other fungi strain which has been used for biological detoxification is *Trichoderma reesei*, but it has been found to degrade compounds such as acetic acid and benzoic and furfural derivatives. Among known physical detoxification methods is extraction with organic solvents such as diethyl ether or ethyl acetate. Both of these solvents have been shown to increase the glucose consumption rate in hydrolysates dramatically, as a result of removal of acetic acid, furfural, vanillin and 4-hydroxybenzoic acid. Chemical methods have been used to detoxify hydrolysates at least for over 70 years. One of the first chemical method that showed great success was increasing the acidity to 9-10 with alkali and readjust to 5.5 with sulfuric acid. Treatment with $Ca(OH)_2$ has been observed to be a more effective way to precipitate toxic compounds than treatment with NaOH. The detoxification by this method is both because of the precipitation of toxic components and the instability of some inhibitors at high pH levels. Furthermore, treatment with reducing agents such as sodium sulfide has a detoxifying effect by decreasing the concentrations of furfural and hydroxymethylfurfural (Palmquist & Hahn-Hagerdal, 2000).

2.6 Production of biofuels by microorganisms

Fermentation

Oxygen is relatively insoluble in water; the equilibrium in distilled water with air temperature of 25°C is 9.6 mg/l. The solubility decreases by increased temperatures. As a result, many habitats are anaerobic. The adaptation of life in an anaerobic environment requires microorganisms utilize organic materials for energy generation by other means than with oxygen dependent respiration (Madigan *et al.*, 2003).

Fermentation is a general term for microbial processes which extract energy (ATP) in the absence of oxygen by catabolizing organic compounds. The electrons removed from the electron donor are used to produce and excrete fermentation products, generated from the original substrate. The fermentation of glucose starts with glycolysis, the conversion of glucose to pyruvate. This process is supplied both by aerobic and anaerobic organisms. Each molecule of glucose (along with 2 ADP and 2 P_i) is converted to a net result of 2 pyruvate molecules, 2 NADH, 2 H^+, 4 ATP and 2 water molecules (Madigan *et al.*, 2003; Nelson & Cox, 2008; Staley *et al.*, 2007).

The fate of pyruvate in further microbial metabolism differs between aerobic and anaerobic microorganisms. Aerobes use the citric cycle and respiratory chain to produce 38 ATP units from each glucose unit, where water and carbon dioxide are end products. However, anaerobes degrade pyruvate in order to regenerate intermediate compounds (most commonly, reducing NADH to NAD^+). At the same time, the substrates serve as electron donors and are oxidized. This results in the production of compounds such as short-chain alcohols, organic acids, CO_2 and hydrogen. For instance, pyruvate can be converted to ethanol in a two-step process, called ethanol fermentation (Figure 12). In the first step, pyruvate is decarboxylized to acetaldehyde by the enzyme pyruvate decarboxylase. In the second step, alcohol dehydrogenase reduces acetaldehyde to ethanol (Staley *et al.*, 2007; Nelson & Cox, 2008).

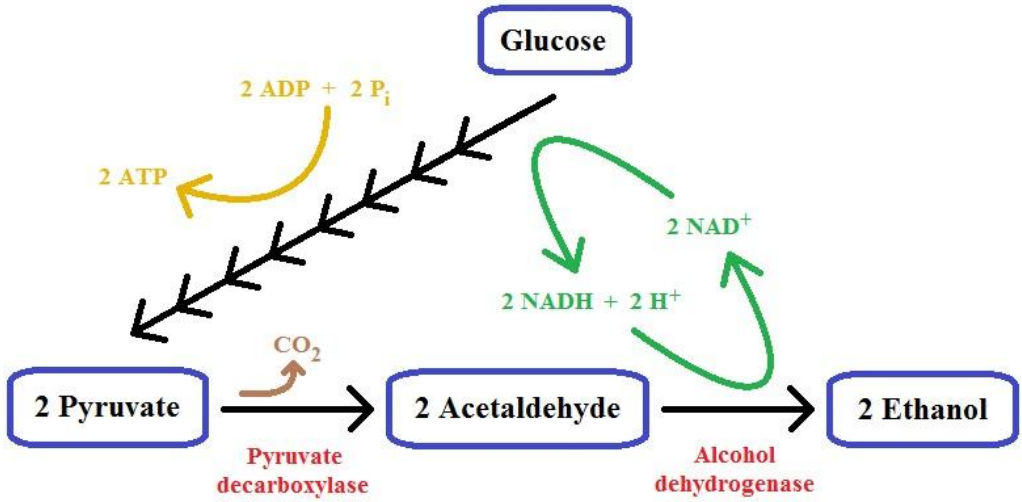

Figure 12. Fermentation of glucose to ethanol. Converting compounds are shown in blue boxes. Intermediate compounds are shown in green, enzymes in red, energy transfer components are shown in orange and the released carbon dioxide is shown in brown color.

Dark fermentation

Biohydrogen can be produced in many different ways, including direct and indirect photolysis, photo-fermentation and dark fermentation (Hung *et al.*, 2011). Among these different techniques, dark fermentation by anaerobic bacteria is most interesting due to the possibility to use various organic waste and wastewater enriched with carbohydrate substrates. That results in a cost effective biohydrogen production which can be processed at high rates. In contrast to photo-fermentation, a dark fermentation process does not need the presence of light. Moreover, valuable metabolites such as butyric, lactic, and acetic acids are produced as by-products. In the last two decades, extensive investigation on dark fermentation has improved both yields and volumetric production rates (Nath & Das, 2004; Lee *et al.*, 2011)

Generally, the production of hydrogen by dark fermentation follows two different pathways. The first one generates hydrogen from NAD(P)H by the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the second one by the enzyme pyruvate ferredoxin oxidoreductase (PFOR)

(Verhaart *et al.*, 2010). The gas produced primarily consists of hydrogen and carbon dioxide. The hydrogen yields depend on the fermentation pathways used by the microorganism and the end products generated. Process conditions such as acidity, hydraulic retention time and gas partial pressure have affect on which metabolic pathways are used and in which ratios. A theoretical maximum of 4 moles of hydrogen can be produced when acetic acid (1) is the end product, but 2 moles when butyric acid (2) is the end product (Figure 13) (Levin *et al.*, 2004):

$C_6H_{12}O_6 + 2\ H_2O \rightarrow 2\ CH_3COOH + 4\ H_2 + 2\ CO_2$ (1)

$C_6H_{12}O_6 + 2\ H_2O \rightarrow CH_2CH_2CH_2COOH + 2\ H_2 + 2\ CO_2$ (2)

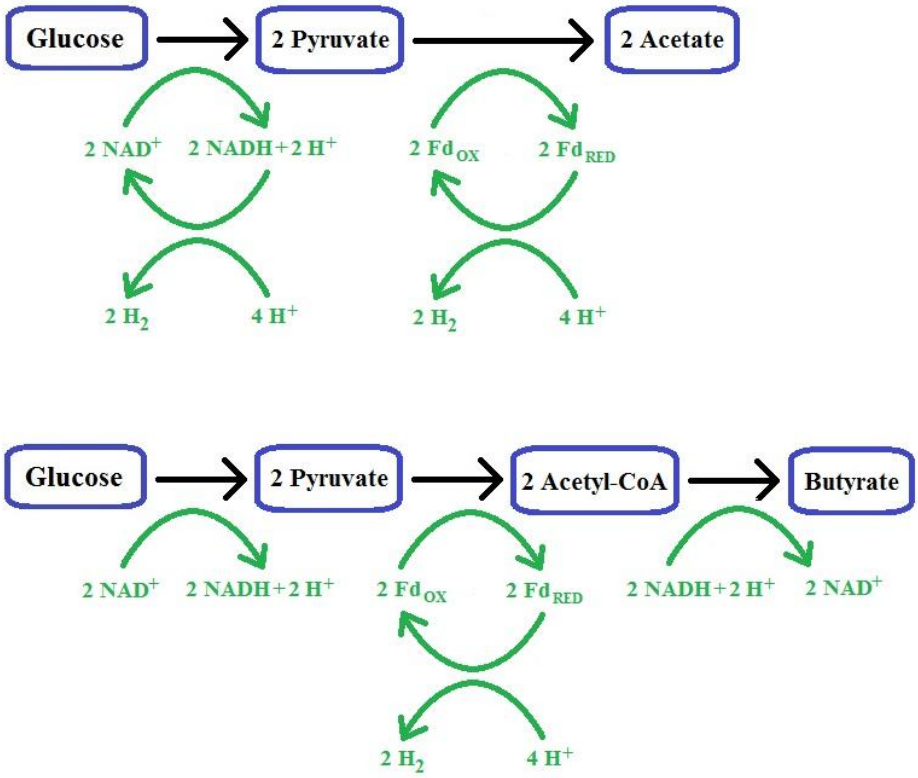

Figure 13. A simplifyed figure for dark fermentation of glucose to acetate and butyrate.

Among dark fermentation mesophilic hydrogen-producing bacteria, species of *Clostridium* and *Enterobacter* are the most widely studied. Dark fermentation investigations on these genera mainly focus on their ability to utilize complex waste, which generally results in a co-culture of the initial dark fermenting bacteria strain and numerous other organisms. In cases where very high yields of hydrogen are observed in such cultures, it might suggest that other suitable dark fermenting bacteria are present in the culture. Isolation of such potential strains are of interest and could evolve the technique of dark fermentation hydrogen production on wastes (Hung *et al.*, 2011). Among thermophilic bacteria, the genera *Caldicellulosiruptor*, *Thermoanaerobacter*, *Thermoanaerobacterium* and *Clostridium* have been thoroughly studied in terms of hydrogen production by dark fermentation (Talluri *et al.*, 2013).

Bioethanol processes from lignocellulosic biomass

In contrast to bioethanol production from simple sugars such as starch or sugar cane (first generation), the conversion form lignocellulosic biomass to ethanol is very complex (second generation). The four most common ways

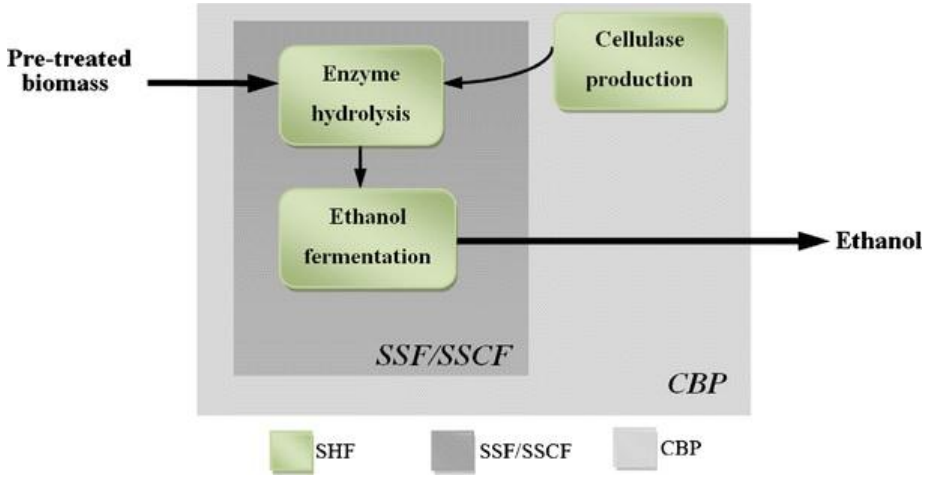

Figure 14. A schematic figure for different processes of lignocellulose conversion to ethanol. For SHF, cellulase production, enzyme hydrolysis and ethanol fermentation are performed in three seperate steps. SSF and SSCF methods combine the enzymatic hydrolysis and ethanol fermentation, whereas CBP combines all the thee processes (Chang & Yao, 2011).

for this process are simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF), separate hydrolysis and fermentation (SHF) and consolidated bioprocessing (CBP) (Figure 14). The difference between these processes is regarding the steps of cellulase production, biomass hydrolysis and sugar fermentation - and how these steps are combined (Chang & Yao, 2011).

SSF and SSCF

The concept of simultaneous saccharification and fermentation (SSF) was initially described in 1977 and the method has been used for small-scale demonstrations, but no commercial plants have to date been built at industrial level. SSF is a process in which the enzymatic degradation and fermentation of the resulting sugars are carried out simultaneously in the same vessel. The key feature of this method is the ability to convert the sugars into ethanol as soon as they are formed by enzymatic hydrolysis, which limits their accumulation in the medium and keeps cellulases away from end product inhibition (Cardona & Sanchez, 2007). Other main advantages are high ethanol yield, productivity and ability to reach relatively high ethanol concentrations (Chang & Yao, 2011).

SSF has disadvantages regarding optimization of process parameters, since hydrolysis and fermentation take place at the same time. Cellulases work optimally at 40-50°C and at pH 4-5. In comparison, hexose fermentation of *S. cerevisiae* is carried out at 30°C at the same pH range. Moreover, the optimal fermentation conditions for pentoses are 30-70°C and pH 5-7 (Cardona & Sanchez, 2007; Olsson & Hahn-Hagerdal, 1996). This means that the selection of microorganism for SSF is strongly limited to conditions favorable for enzymatic hydrolysis.

Simultaneous saccharification and co-fermentation (SSCF) is very similar to SSF. The difference is that SSCF does in contrast to SSF include pentose fermentation, which makes the process more efficient. Although the yeast *S. cerevisiae* is the most commonly used microorganism for ethanol production, thermophilic bacteria is probably more suitable for these methods. Among thermophilic bacteria are strains that have optimal growth temperatures close to the optimal temperatures of hydrolysis. Moreover, thermophilic bacteria strains are known for broad utilization spectra, which is

essential for the SSCF process. Strains of *T. saccharolyticum* have for instance been reported as suitable for SSCF. (Chang & Yao, 2011).

SHF

Separate hydrolysis and fermentation (SHF) is a process where the biomass hydrolysis and sugar fermentation is performed in two discrete steps. This method has the advantage that both hydrolysis and fermentation can be carried out at optimum environmental conditions, which means that the selection of microorganisms is not as dependent on certain optimum growth temperatures (Chang & Yao, 2011).

The accumulation of glucose in the hydrolysis step is a major drawback of SHF (Chang & Yao, 2011). Since sugars are generally more inhibitory for conversation process than ethanol, SHF does not reach as high rates and ethanol yields as SSF/SSCF (Cardona & Sanchez, 2007). Moreover, the need for two bioreactors to perform hydrolysis and fermentation increases the cost of the SHF method in comparison to the simultaneous methods (Alfani *et al.*, 2000).

CBP

In 1996, a new concept for bioethanol process was described. Consolidated bioprocessing (CBP), also called direct microbial conversion (DMC), is a process that combines enzyme production, saccharification of cellulose and hemicellulose, and fermentation, in one single step. This method is promising for future bioethanol for economical reasons, both regarding the process itself and capital investment on equipment (Hasunuma & Kondo, 2012). The most important part is that the process includes enzyme production, which is a major part of the cost of the SSF, SSCF and SHF methods (Cardona & Sanchez, 2007). In 2005, bioethanol production by SSCF was estimated over four times more expensive than by CBP (Lynd *et al.*, 2005).

However, the requirements for microorganisms carrying out the CBP process are more than for the other methods described above. CBP-microorganisms must produce enzymes which effectively hydrolyze lignocellulosic polysaccharides to fermentable sugars and convert the sugars to ethanol efficiently, regarding ethanol concentrations, yields and productivity. Both pentoses and hexoses must be utilized. Moreover,

resistance to ethanol, fermentation inhibitors and stressful environments such as high osmotic pressure, low pH, high temperature, low nutrition and fluctuating processes is required. No microorganisms with these characteristics are currently available (Hasunuma & Kondo, 2012).

In light of the advantages of CBP, it is hoped that suitable CBP-microorganisms will be developed in near future by microbial engineering. Two main approaches for such engineering have been described; the native cellulolytic strategy and the recombinant cellulolytic strategy. The native cellulolytic strategy is based on engineering naturally occurring cellulolytic microorganisms to improve product-related properties such as yield and titer, whereas the recombinant cellulolytic strategy involves engineering of non-cellulolytic organisms that exhibit promising product properties so that they express a heterologous cellulase system that enables cellulose utilization (Lynd *et al.*, 2005).

Many different microorganisms have been used in studies trying to generate a suitable CBP strain by genetic modifications. *Clostridium thermocellum* and *Thermoanaerobacterium saccharolyticum* are two commonly used bacteria strains for the native cellulolytic strategy. Moreover, the fungi *T. reesei* has some interesting features for this approach, including production of cellulases in very high quantities. The focus has been on increasing the ethanol yield, eliminate byproducts, improve ethanol tolerance, and introduction of new and desirable metabolic pathways for assimilating most or all lignocellulose sugars. Among microorganisms that have been used for the recombinant cellulolytic strategy are the bacteria *Z. mobilis*, *E. coli* and *K. oxytoca*, as well as the yeast *S. cerevisiae*, *P. tannophilus*, *P. stipitis* and *C. shehatae*. The aim of genetic engineering for the recombinant cellulolytic strategy is mainly to introduce functional production and secretion of a variety of endo- and exoglucanases, growth in lignocellulosic biomass as a sole carbon source and assimilation and fermentation of all sugars derived from lignocellulose (Xu *et al.*, 2009).

Ethanol production by *S. cerevisiae*

The yeast *Saccharomyces cerevisiae* is the conventional microorganism for first generation bioethanol production (Sanchez *et al.*, 2010). The natural ability of fast ethanol accumulation and high ethanol tolerance is believed to

have helped *S. cerevisiae* in competition with other organisms by inhibiting their growth (Piskur *et al.*, 2006). *S. cerevisiae* shows up to 93% of the theoretical yield (1 mol glucose converted to almost 2 moles ethanol) when optimal conditions are used (Bai *et al.*, 2008). Among other advantages of the yeast is high tolerance to acids, tolerance to the wide spectrum of inhibitors generally present in hydrolysates and tolerance to high osmotic pressure. *S. cerevisiae* is able to ferment hexoses such as glucose, mannose and galactose to ethanol and has been used for first generation bioethanol production for a long time (Hahn-Hagerdal *et al.*, 2007). The major drawback of *S. cerevisiae* is its lack of ability to ferment pentose sugars.

As mentioned earlier, the potential of *S. cerevisiae* to produce ethanol in high yields has led to genetic engineering studies trying to express genes from other organisms which make the yeast capable of degrading the broad variety of sugars present in pretreated lignocellulosic biomass. Various metabolic engineering strategies have been employed to express both fungal and bacterial genes encoding either D-xylose or L-arabinose pathways in the yeast, since these sugars account for a large proportion of hydrolyzed hemicellulose. Despite these efforts, heterologous expression of these genes have not been sufficient to enable the fermentation of pentoses to ethanol (Sanchez *et al.*, 2010).

Although *S. cerevisiae* is best known for its ability of producing high yields of ethanol, it can also be turned into an effective producer of a group compounds referred to as "advanced biofuels" by genetic modifications. These compounds include the high alcohols such as 1-butanol and isobutanol, sesquiterpenes such as farnesene and bisabolene and fatty acid ethyl esters such as biodiesel. Advanced biofuels might be promising due to high energy density, but most of them need further investigation (Buijs *et al.*, 2013).

Production of biofuels by thermophilic bacteria

In recent years, increased interest has been in using thermophilic bacteria for biofuel production. This is a result of many advantageous factors which are characteristic for these microorganisms. For instance, many thermophilic bacteria have shown to possess unique cellulolytic and hemicellulolytic systems and are considered as potential sources of highly active and thermostable enzymes for efficient biomass hydrolysis. In contrast to the

yeast *S. cerevisiae*, thermophilic bacteria have commonly a broad substrate spectrum, and are able to ferment the majority of the pentoses present in hydrolysates. Moreover, biofuel production at high temperatures decreases the risk of contamination. These properties do not only offer an opportunity to produce renewable biofuels in a sustainable way, but can also be very cost-effective since agricultural residues and wastes can be utilized and less agricultural land is needed. The most important challenge to solve is to carry out genetic engineering on suitable thermophilic strains, for instance in order to decrease the inhibitory compounds sensitivity and enhance metabolic pathways (Chang & Yao, 2011).

Bioethanol production

As described earlier, bioethanol has been produced and used as a fuel for a very long time. Even though the industry has mainly been based on first generation production (Table 2), production from complex biomass is continuously gaining increased attention (Table 3). Three decades ago the earliest available data on bioethanol production from lignocellulosic biomass by thermophilic bacteria was published. The most promising results from this study were on *Thermoanaerobacter ethanolicus* (strain JW200), using hemicellulose from birch- and beechwood sources (batch cultures), with yields of 0.81 mol ethanol per mol xylose (Wiegel *et al.*, 1983).

Clostridium thermocellum is another thermophilic bacteria that has been studied thoroughly in terms of ethanol production. The strain has been shown to produce at least 7.2 mM ethanol per g avicel, but the theoretical maximum is 11.1 mM (Sveinsdottir *et al.*, 2011). Rani and co-workers studied ethanol production on various carbon sources and observed similar yields for Whatman paper, paddy straw, sorghum stover corn stubs, in which alkali pretreatment was used. The strain produced approximately 1.44 mol ethanol per mol carbon source (Rani *et al.*, 1997). Baluso and co-workers used a statistical methodology called Plackett-Burman design to screen 23 nutrients for ethanol production by the *C. thermocellum* strain SS19. The highest ethanol yields were observed on Whatman paper, 1.48 mol ethanol per mol substrate. Other nutrients that were of great importance according to the statistical methodology (based on productivity, availability and cost-efficiency) were corn steep liquor, cysteine HCl, magnesium chloride and

Table 2. Ethanol production from various sugars with thermophilic bacteria. Ethanol yields are shown in mM per g substrate degraded. Substrate concentrations and incubation temperature is shown. Biomass: (GLU) glucose, (XYL) xylose, (CBS) cellobiose, (SUC) sucrose.

Organism	Substrate	Substr. conc. (g/L)	Ethanol yield (mM / g sugar)	Temp. (°C)	Ref.*
T. thermohydrosulfuricus	GLU	5.0	1.60	60	A
T. finnii	GLU	NA	1.45	60	B
T. finnii	XYL	NA	1.76	60	B
C. thermocellum	CBS	2.6	1.60	60	C
T. ethanolicus	SUC	15.0-30.0	1.80-3.60	65	D
T. thermohydrosulfuricus	SUC	15.0-30.0	1.10-3.00	65	D
Thermoanaerobacter ap 65-2	SUC	15.0-30.0	1.30-3.20	65	D
Thermoanaerobacterium AK_{17}	GLU	3.6	1.50	60	E
Thermoanaerobacterium AK_{17}	XYL	3.0	1.10	60	E
Mixed culture	GLU	5.0	1.53	70	F
Mixed culture	XYL	2.0	1.60	70	G
Enrichment cultures	GLU	9.0	1.34	50-75	H
Enrichment cultures	XYL	7.5	1.30	50-75	H
T. ethanolicus	GLU	5.0	1.20-1.30	65	I
T. ethanolicus	XYL	5.0	1.00-1.20	65	I

*References: (A) Lovitt *et al*. (1984), (B) Fardeau *et al*. (1996), (C) Knutson *et al*. (1999) (D) Avci *et al*. (2006), (E) Sveinsdottir *et al*. (2009), (F) Zhao *et al*. (2009b), (G) Zhao *et al*. (2010), (H) Orlygsson *et al*. (2010), (I) He *et al*. (2010).

Table 3. Ethanol production from various biomass sources with thermophilic bacteria. Ethanol yields are shown in mM per g substrate degraded. Substrate concentrations and incubation temperature is shown. Biomass: (WHL) wood hydrolysate, (WHP) Whatman paper, (AVI) avicel, (XYL) xylan, (WST) wheat straw, (CST) corn stover, (BNC) been card hydrolysate, (CEL) cellulose, (GRA) grass, (PAP) paper, (NPG) Napier grass, (HPS) hemp stem.

Organism	Biomass	Substr. conc. (g/L)	Ethanol yield (mM / g sugar)	Temp. (°C)	Ref.*
T. ehanolicus	WHL	8.0	3.30-4.50	70	A
C. thermocellum	WHP	8.0	7.20-8.00	60	B
C. thermocellum	AVI	8.0	6.50-7.20	60	B
Thermophilic strain A3	XYL	10.0	5.43	70	C
T. saccharolyticum	XYL	10.0	6.30	60	C
T. mathranii	WST	60.0 (6.7)*	2.61	70	D
T. mathranii	WST	60.0	5.30	70	E
Thermoanaerobacter BG1L1	CST	25.0-150.0	8.50-9.20	70	F
Thermoanaerobacter BG1L1	WST	30.0-120.0	8.50-9.20	70	G
Thermoanaerobacter BG1L1**	CST	25.0-150.0	8.50-9.20	70	G
T. ehanolicus	BNC	10.0	1.80	60	H
Clostridium sp.	BNC	10.0	0.85	60	H
Thermoanaerobacterium sp.	BNC	10.0	0.90	60	H
Thermoanaerobacterium AK_{17}	CEL	7.5	5.81	60	I
Thermoanaerobacterium AK_{17}	GRA	7.5	2.91	60	I
Thermoanaerobacterium AK_{17}	PAP	7.5	2.03	60	I
C. thermocellum	NPG	2.0-40.0	0.80-3.90	60	J
C. thermocellum	AVI	10.0	0.70	60	J
Clostridium sp. AK_1	HPS	5.0	3.5	45	K

*References: (A) Wiegel *et al*. (1983), (B) Rani *et al*. (1997), (C) Ahring *et al*. (1996), (D) Ahring *et al*. (1999), (E) Klinke *et al*. (2001), (F) Georgieva *et al*. (2007a), (G) Georgieva *et al*. (2008), (H) Maiyazaki *et al*. (2008), (I) Sveinsdottir *et al*. (2009), (J) Lin *et al*. (2010), (K) Orlygsson (2012).

ferrous sulfate (Balusu *et al.*, 2004). All these studies with *C. thermocellum* had biomass concentrations below 8.0 g/L.

Orlygsson investigated *Clostridium* sp. AK_1 which was isolated from a hot spring in Iceland. The strain was able to utilize several different hydrolysates; Whatman paper, newspaper, timothy grass, hemp leaves, hemp stem and Barley straw. When no chemical pretreatment was used the highest yields were observed on Whatman paper, 1.33 mol ethanol per mol biomass. Moreover, the ethanol production from the hydrolysates was enhanced by pretreatment with both dilute acid and alkali. The highest yields were observed on alkali pretreated hemp stem, 0.63 mol ethanol per mol biomass (Orlygsson, 2012).

Thermoanaerobacterium saccharolyticum is able to utilize all naturally occurring sugars derived from the polymers in lignocellulosic biomass (Olson *et al.*, 2012). Shaw and co-workers genetically engineered the strain where they eliminated the production of acetic acid and lactic acid. The strain used over 99% of the fed xylose at initial sugar concentrations up to 70 g/L, and had a mean ethanol yield of 1.51 mol ethanol per mol xylose. These results were both observed for batch and continuous cultures (Shaw *et al.*, 2008). Ahring et al. (1996) investigated two different *T. saccharolyticum* strains and their ability to produce ethanol from the hemicellulose fraction of wheat straw hydrolysates. The strains, A3 and HG8, produced 1.14 and 0.98 mol ethanol per mol xylan, respectively (Ahring *et al.*, 1996).

Sveinsdottir and co-workers studied ethanol production from hydrolysates by *Thermoanaerobacterium* sp. AK_{17}. Batch cultures with a solid loading of 7.5 g/L were used at the optimal temperature of 60°C and pH 6. No chemical pretreatments were used. The highest yields were observed on Whatman paper, 1.05 mol ethanol per mol biomass (Sveinsdottir *et al.*, 2009).

The ethanol production of *Thermoanaerobacter mathranii*, isolated from Hveragerdi in Iceland in 1993, was investigated by Ahring and co-workers (1999). Wheat straw (60 g/L) was pretreated with different amounts of sodium carbonate, resulting in various amounts of sugars released (3.5 to 9.9 g/L). The strain produced approximately 0.23 mol ethanol per mol sugar (Ahring *et al.*, 1999). Furthermore, Klinke and co-workers investigated the same strain in order to assess the effect of different hydrolysate concentration and inhibitory compounds on the fermentation of wheat straw. Alkaline wet

oxidation was used as a pretreatment method. No inhibition of ethanol production was observed when cultivated on the hydrolysate with 4 g/L xylose. Nine phenols and 2-furoic acid were identified in the hydrolysate. The concentrations of these inhibitory compounds was increased to analyze the inhibitory effect. When the concentrations of each of these compounds was increased to 2 mM, neither a growth inhibition nor decreased ethanol yields were observed. A severe inhibition was observed when the concentrations of inhibitors was increased to 10 mM. Phenolic aldehydes had a more inhibitory effect than phenol ketones (Klinke et al., 2011).

Cripps and co-workers deleted lactate dehydrogenase and pyruvate formate lyase pathways of the bacteria *Geobacillus thermoglucosidasius* in order to direct the end product formation to ethanol. The enzyme pyruvate dehydrogenase was upregulated. This resulted in an ethanol yield of 1.54 mol ethanol per mol glucose, up to 92% of theoretical value (Cripps *et al.*, 2009).

In 2007 and 2008, the highest ethanol yield that had been found on complex biomass by thermophilic bacteria was reported, using *Thermoanaerobacter* strain BG1L1. Corn stover and wheat straw was pretreated in two different ways, with acid and wet oxidation, resulting in ethanol yields up to 1.65 mol ethanol per mol sugars in the hydrolysates (Georgieva *et al.*, 2007a; Georgieva *et al.*, 2008).

Biohydrogen production

In contrast to bioethanol, which has been used as a fuel for a very long time, biohydrogen has yet to be used as a widely distributed fuel. The data on second generation biohydrogen production has exploded in the last few years. Table 4 shows results from several investigations on biohydrogen production from simple sugars, whereas Table 5 includes studies on the production from complex biomass.

Since optimum temperature and hydrogen production of bacteria is generally correlated, species of the genera *Caldicellulosiruptor* and *Thermotoga* have been observed to be the most efficient biohydrogen producers. Ivanova and co-workers published results on hydrogen production of *C. saccharolyticus* on several different hydrolysates (e.g. wheat straw, maize leaves, sweet sorghum plant, sweet sorghum juice and *Silphium trifoliatum* leaves), using batch cultures. The highest hydrogen yield was observed on wheat straw (1% TS), 3.80 mol hydrogen per mol glucose. On

Table 4. Hydrogen production from sugars with thermophilic bacteria. Hydrogen yields are shown in mol per mol substrate degraded. Substrate concentrations, cultivation method and incubation temperature is shown. Substrate: (MAL) maltose, (GLU) glucose, (SUC) sucrose, (XYL) xylose, (LAC) lactose.

Organism	Substrate	Cultiv. method	Substr. conc. (g/L)	H_2 yield (mol H_2 / mol glucose equiv.)	Temp. (°C)	Ref.*
P. furiosus	MAL	Cont.	0.22	2.90	98	A
T. maritima	GLU	Batch	0.1	4.00	80	B
T. elfii	GLU	Cont.	10.0	3.30	65	C
C. saccharolyticus	SUC	Cont.	10.0	3.30	70	C
T. tencongensis	GLU	Cont.	4.5	4.00	70	D
C. saccharolyticus	GLU	Cont.	4.0	3.60	70	E
T. thermosaccharolyticum	SUC	Batch	20.0	2.53	60	F
T. thermosaccharolyticum	GLU	Batch	10.0	2.42	60	G
T. thermosaccharolyticum	XYL	Batch	10.0	2.19	60	G
T. neapolitana	GLU	Batch	2.5	3.85	77	H
C. saccharolyticus	GLU	Cont.	10.0	3.00	70	I
T. thermosaccharolyticum	XYL	Batch	12.2	2.37	60	J
T. neapolitana	GLU	Cont.	5.0	3.85	80	K
C. owensis	GLU	Cont.	10.0	3.80	70	L
C. owensis	XYL	Cont.	10.0	2.70	70	L
C. thermolacticum	LAC	Batch	10.0	1.80	58	M
Clostridium AK17	GLU	Batch	3.6	2.21	50	N
Clostridium AK17	XYL	Batch	3.0	2.55	50	N

*References: (A) Schicho et al. (1993), (B) Schroder et al. (1994), (C) van Niel et al. (2002), (D) Soboh et al. (2004), (E) Vrije et al. (2007), (F) O-Thong et al (2008), (G) Ren et al. (2008), (H) Munro et al. (2009), (I) Willquist et al. (2009), (J) Cao et al. (2010), (K) d'Ippolito et al. (2010), (L) Zeidan & van Niel (2010), (M) Collet et al. (2003), (N) Almarsdottir et al. (2010).

Table 5. Hydrogen production from various biomass sources with thermophilic bacteria. Hydrogen yields are shown in mol per mol substrate degraded. Substrate concentrations, cultivation method and incubation temperature is shown. Biomass: (PSL) paper sludge, (MHL) miscanthus hydrolysate, (WST) wheat straw, (SSP) sweet sorghum plant, (SBG) sugarcane bagasse, (MLV) maize leaves, (GRA) grass, (PAP) paper, (BLS) Barley straw, (CST) Corn stover. All experiments are batch cultures.

Organism	Biomass	Substr. conc. (g/L)	H_2 yield (mol H_2 / mol glucose equiv.)	Temp. (°C)	Ref.*
C. Saccharolyticus	PSL	8.4	3.70	70	A
T. Thermosaccharolyticum	MHL	10.0	3.40	72	B
T. Neapolitana	MHL	14.0	3.20	80	B
C. Saccharolyticus	WST	20.0	3.80	70	C
C. Saccharolyticus	SSP	30.0	1.75	70	C
C. Saccharolyticus	SBG	15.0	2.30	70	C
C. Saccharolyticus	MLV	8.0	3.67	70	C
Clostridium AK_{14}	CEL	5.0	0.10-0.20	50	D
Clostridium AK_{14}	HPS	5.0	0.60-0.70	50	D
Clostridium AK_{14}	HPL	5.0	0.20-0.40	50	D
Clostridium AK_{14}	GRA	5.0	0.80-0.90	50	D
Clostridium AK_{14}	PAP	5.0	0.10-0.40	50	D
Clostridium AK_{14}	BLS	5.0	0.70-0.80	50	D
*T. Thermosaccharolyticum*W16	CST	10**	2.70	60	E

*References: (A) Kadar et al. (2004), (B) Vrije et al. (2009), (C) Ivanova et al. (2009), (D) Almarsdottir et al. (2010), (E) Ren et al. (2010).

** = Sugar concentration

pretreated maize leaves, 3.67 mol hydrogen per mol glucose was observed. For comparison, another set of experiment contained non-sterile biomass. All the hydrolysate types showed reduced ethanol yield when the sterilization step was let undone (Ivanova *et al.*, 2009).

Hydrogen production of *Thermotoga neopolitana* has been described in several studies. d'Ippolito and co-workers used continuous culture of the bacteria with glucose (5 g/L) as carbon source and observed 3.85 mol hydrogen per mol glucose (d'Ippolito *et al.*, 2010). Another group of scientists observed the same yields for a batch culture with initial glucose concentration of 2.5 g/L (Munro *et al.*, 2009).

Among other thermophilic bacteria that has been investigated for biohydrogen production is *Thermoanaerobacterium thermosaccharolyticum*. Cao and co-workers used strain *T. thermosaccharolyticum* W16 for investigations on corn stover, pretreated and hydrolyzed by dilute sulfuric acid. The optimum hydrolysis conditions were 1.69% sulfuric acid with a pretreatment reaction time of 117 minutes. For that conditions, strain W16 produced 2.24 mol hydrogen per mol sugar (Cao *et al.*, 2009). Another group of scientists used the same strain to investigate the hydrogen yield on a mixture of the two most common monomers derived from lignocellulosic biomass; glucose and xylose. The observed hydrogen yield on pure glucose and xylose was 2.42 mol hydrogen per mol glucose and 2.19 mol hydrogen per mol xylose. The results for the mixtures of the two sugars showed a stable gradient where the yield correlated to the concentration of glucose. Moreover, the highest production rate was observed on pure glucose (Ren *et al.*, 2008).

Almarsdottir and co-workers investigated hydrogen production of the moderate thermophile *Clostridium* strain AK14, using hydrolasates from hemp leaves, hemp stem, timothy grass, barley straw, newspaper and Whatman paper. Both dilute acid and alkali pretreatments were used. The highest ethanol yields were observed on dilute acid (0.75% sulfuric acid) pretreated timothy grass, 1.01 mol hydrogen per mol sugars. Both dilute acid pretreatment and alkali pretreatment increased the hydrogen yields in this experiment (Almarsdottir *et al.*, 2010).

Pattra and co-workers investigated the hydrogen production ability of *Clostridium butyricum* from sugarcane bagasse hydrolysate, containing 11

g/L glucose, 11 g/L xylose and 2 g/L arabinose. The best hydrogen yields were obtained with an initial sugar concentration of 20 g/L; 1.73 mol hydrogen per mol sugar (Pattra *et al.*, 2008).

Kongjan & Angelidaki used mixed cultures which, according to phylogenetic analysis, included the thermophilic hydrogen producing species *Caldanaerobacter subterraneus*, *Thermoanaerobacter subterraneus* and *Thermoanaerobacterium thermosaccharolyticum*. The cultures originated from methanogenic sludge from a potato factory, and were set up for different continuous reactors (UASB, CSTR and AF) with hemicellulose rich wheat straw (4.4% TS) as energy source. The highest hydrogen yields were observed in the UASB reactor, corresponding to 1.70 mol hydrogen per mol glucose. One of the main conclusions from this study was the great importance of reactor configuration for enhancing and stabilizing hydrogen production (Kongjan & Angelidaki, 2010).

3 Materials and methods

3.1 Organisms and sampling sites

At the beginning of the research a total number of 125 bacteria strains were screened for end product formation from glucose, cellulose and avicel. This resulted in selection of four strains that were chosen for further investigation. All these strains were isolated from hot spring areas at two sampling sites; Grændalur in the Hengill area in South-West Iceland, and the Krafla area in North-East Iceland. The samples were collected in 2007 and 2009 and enriched on various carbon sources. The isolation method has been described earlier (Orlygsson & Baldursson, 2007). After the isolation, the strains were stored in 30% glycerol at -20°C.

The four strains that were chosen from screening experiments at the beginning of the research were given the names J1, J2, J3 and J4. Table 6 shows the environmental conditions at the sampling sites as well as culture conditions used for experiments.

Table 6. Strain identity names, the temperature and acidity of the sampling sites and the temperature and acidity used for isolation.

Strain	Site temperature (°C)	Site acidity (pH)	Isolation temperature (°C)	Isolation acidity (pH)
J1	69	7.5	70	7.0
J2	50	7.4	50	7.0
J3	50	7.7	50	7.0
J4	65	8.0	65	8.0

3.2 Medium

The same type of medium was used for all experiments; the content is shown in Table 7. The medium was prepared by mixing the distilled water, yeast extract, buffer and resazurin in an Erlenmeyer flask, heat it up to the boiling point and boil it until the color of the solution turned sharp pink. The medium was sparged with nitrogen while cooling down to room temperature, and then the pH level was adjusted. For all experiments done with strains J1 and J2 the medium with pH 7.0 was used whereas pH 8.0 was used for strains J3 and J4. After pouring the medium into serum bottles they were closed and the gas phase was sparged with nitrogen. Finally the medium was autoclaved for 60 minutes.

Table 7. The contents of the basal medium

Compound	Amount in 1 liter of med
Distilled H_2O	885 ml
Buffer	50 ml
Yeast extract	2 g
Resazurine	5 mL
NaCl*	0,3 g
NH_4Cl*	0,3 g
$CaCl_2 \cdot 2H_2O$*	0,11 g
$MgCl_2 \cdot 6H_2O$*	0,1 g
$FeCl \cdot 4H_2O$*	2 mg
EDTA*	0,5 mg
$CuCl_2$*	0,03 mg
H_3BO_3*	0,05 mg
$ZnCl_2$*	0,05 mg
$MnCl_2 \cdot 4H_2O$*	0,05 mg
$(NH_4)_6MO_7O_{24} \cdot 4H_2O$*	0,05 mg
$AlCl_2$*	0,05 mg
$COCl_2 \cdot 6H2O$*	0,05 mg
$NaHCO_3$**	0,72 g
$C_3H_7NO_2S \cdot HCl \cdot H2O$**	5 mg
$Na_2S \cdot 9H2O$**	0,24 g
Vitamin solution***	1 mL

* Contents of solution C_1

** Contents of solution C_2

Some of the remaining components of the medium were mixed and dissolved in distilled water. This solution is called C_1 (trace elements, vitamins and salts) and was added to each serum bottle just before the start of an experiment. Another solution called C_2, containing additional buffer, $NaHCO_3$, and the reducing compounds cysteine chloride and sodium sulfide were added after solution C_1. All solutions added to the medium after autoclaving were filter sterilized.

3.3 Batch cultures

All experiments in this research were performed as batch cultures. They were performed in serum bottles of three different sizes; 24.5 ml, 58 ml and 117.5 ml. The bottles are closed with butyl rubbers and sealed with aluminum caps, preventing any ventilation between the gas phase of the culture and the environment. The three different sizes of serum bottles, the butyl rubbers and the aluminum caps are shown in Figure 15.

The inoculum used for the batch cultures were derived from cultures stored in 30% glycerol at -20°C (sampling described in chapter 3.1). Each strain stored in a freezer was re-inoculated several times in order to get a dense culture. The inoculation was performed at the same temperature and acidity the strains were originally enriched at, with 20 mM glucose as a carbon source. The inoculum was usually taken out from the incubator during late exponential growth phase and kept at room temperature until it was used for batch experiments.

Figure 15. The different sized serum bottles the butyl rubbers and aluminum caps used to close the bottles are shown in front.

Strains J1 and J4 were always incubated at 65°C and strains J2 and J3 at 50°C. All experiments were performed in duplicate and the results are mean values of the two replicates, unless exceptions are mentioned in the corresponding method description.

3.4 Biomass

Two experiments with hydrolysates as carbon source were performed. In the first one, six different types of hydrolysates were used; Whatman paper, inked newspaper, hemp leaf, hemp stem, straw and timothy grass.

In the second experiment, two very similar samples of timothy grass were used. There was a slight difference in the grass composition, as shown in Table 8. Both of these grass samples were harvested on June 26th 2012 and fertilized on July 4th 2012 with 34 kg N/ha (inorganic), 800 kg/ha of compost and 6500 kg/ha of cow manure.

Table 8. The composition of the two timothy grass types.

	Akramýri	***Tjarnarspilda***
Timothy (*Phelum pratense*)	80-90%	80-90%
Meadow grass	Trace	10-15%
Meadow foxtail	–	Trace
Tufted Hair-grass	Trace	–
Dandelions	Trace	Trace
Northern Dock	–	Trace
Cerastium fontanum	Trace	–
White clover	–	Trace

3.5 Analytical methods

Hydrogen was measured by a Perkin Elmer gas chromatograph as previously described (Orlygsson & Baldursson, 2007). The analysis of ethanol and volatile fatty acids was performed by a Perkin Elmer Clarus 580 gas chromatograph using an FID detector with a 30 metres DB-FFAP capillary column (Agilent Industries Inc, Palo Alto, CA, US). Glucose and xylose were

analyzed according to (Leyva *et al.*, 2008) and (Eberts *et al.*, 1979). The final pH of the cultures was measured by a Thermo Scientific 4 star pH meter.

For the later experiments performed on timothy grass, high performance liquid chromatography (HPLC) with a refractive index detector was used to measure the amounts of different sugars in the samples. The column used for the HPLC runs was a Bio-Rad Aminex HPX-87H (300 mm × 7,8 mm) and the column oven temperature set to 63.5°C. A 0.05 M H_2SO_4 solution was used as eluent, with a flow rate of 0.6 ml/min. Each sample was diluted up to 5 times.

3.6 Experiments

Screening of the University of Akureyri culture collection

Each strain in the culture collection derived from the sampling trips in 2007 and 2009 were cultivated in this first experiment.

Small volume (24.5 ml) bottles were used with 10 ml liquid volume, which results in a liquid/gas (L/G) ratio of 0.69. Each culture had a carbon source of 20 mM glucose. The cultivation was stopped after 96 hours. This experiment was performed without parallels (single).

After this initial growth experiment on glucose another screening experiment was performed. Three different carbon sources were used; glucose (20 mM), and two types of cellulose (cellulose powder and avicel, both 3 g/l). For the cellulose and avicel cultures, the polymers were added as powder to the medium, before the media was boiled.

Since 1 ml was taken from each bottle before growth (T_0), containing glucose, the total liquid volume was 9 ml and the L/G ratio was 0.58. The total liquid volume for the cellulose and avicel cultures was 9.8 ml, which corresponds to a L/G ratio of 0.67. The cultivation was stopped after 168 hours of incubating.

Phylogeny

The 16S rRNA sequence analysis was carried out as described earlier (Vesteinsdottir *et al.*, 2011). The NCBI database was used to compare the results with other known strains by the BLASTN tool. The programs BioEdit (Hall, 1999), CLUSTAL_X (Thompson *et al.*, 1997) and TreeView were used to display a phylogenetic tree, using *Caloramator viterbensis* as an outgroup. The traditional gram staining method was used to analyze the cell wall type of the strains.

Substrate utilization spectrum

To investigate the substrate spectrum of the strains, 24.5 ml bottles were used. A total number of 22 different carbon sources were used. For five carbon sources (xylan, starch, CMC, cellubiose and avicel), the solid powder was added to the medium (3 g/l) before it was boiled. For the others, one molar stock solution of the carbon source was made by dissolving it in distilled water and filtering them into autoclaved, anaerobic bottles. This was done for arabinose, cellulose, fructose, galactose, glucose, glycerol, lactose, maltose, mannose, pyruvate, raffinose, rhamnose, serine, sucrose, threonine, trehalose and xylose. The initial concentration of each carbon source in this experiment was 20 mM. The total liquid volume of each culture was 10 ml, corresponding to a L/G ratio of 0.69. The incubation time of the cultures was 168 hours.

Experiment with different initial concentrations of glucose for strains J1, J2 and J4

To investigate the effects of different initial glucose concentrations on both yields and substrate utilization, seven different concentrations (5, 10, 20, 30, 50, 100 and 200 mM) were used. Large volume (117.5 ml) bottles were used with 60 ml medium. The L/G ratio in the bottles was 1.04. The incubation time for this experiment was 168 hours. Strain J3 was not used due to problems with reactivating the strain from older cultures, which prevented it from being used for several experiments of this study.

Effect of liquid/gas (L/G) ratio on end product formation

In this experiment 117.5 ml bottles were used and glucose (20 mM) was the carbon source. The cultures were incubated for 168 hours.

The cultures were prepared by using six different total liquid volumes - yielding six different L/G ratios. The total liquid volumes were 2 ml (L/G ratio of 0.02), 5 ml (L/G ratio of 0.04), 10 ml (L/G ratio of 0.09), 30 ml (L/G of 0.34), 60 ml (L/G ratio of 1.04) and 90 ml (L/G ratio of 3.27). One control culture was made for each total liquid volume of each strain (no glucose added as a carbon source).

Heat resistance of strains J1, J2 and J4

Three strains were tested for heat resistance in the following way. Each strain was inoculated to a 58 ml bottle, with 20 ml of liquid volume and 20 mM glucose, and was incubated until late exponential growth phase was reached. This took a little less than 24 hours for strain J2 but almost 48 hours for strains J1 and J4.

After cooling down to room temperature, the bottles were put into a 100°C incubator for a heat shock. A T_0-sample was taken, and then a sample from each bottle every 10 minutes. The last sample was taken 100 minutes after the heat shock started.

Each sample was directly injected into a fresh glucose (20 mM) containing media of the same size as before and incubated at the optimum temperature for the corresponding strains. They were allowed to grow for 120 hours before samples were taken and analyzed.

Ethanol tolerance of strains J1, J2 and J4

Small volume (24.5 ml) bottles were used for this experiment containing 8.8 ml of medium, 0.2 ml of inoculum, 1 ml of ethanol solution and 0.2 ml of glucose (20 mM). Different stock solutions of ethanol were made in order to give a final concentration of 0, 0.2, 0.4, 0.8, 1.6, 3.2, 4.2, 5.2, 6.2, 7.2 and 8.2% (vol/vol). The cultures were stopped after 120 hours incubation.

Since the ethanol tolerance of the strains could only be roughly estimated, another experiment was set up to find out a more accurate tolerance level. This second round of experiment was set up in the same way

as before, but the initial ethanol concentrations were 1.4, 1.6, 1.8, 2.0, 2.2, 2.4, 2.6, 2.8, 3.0, 3.2, 3.4, 3.6, 3.8 and 4.0 percent. These cultures were incubated for 168 hours.

A third round of ethanol tolerance experiment was needed because strain J1 tolerated the highest ethanol concentration (4.0%) used. This experiment had the same setup as the others, but the initial ethanol concentrations were 4.0, 4.2, 4.4, 4.6, 4.8, 5.0, 5.2, 5.4, 5.6, 5.8, 6.0, 6.2, 6.4, 6.6, 6.8 and 7.0 percent. These cultures were incubated for 168 hours.

Fermentation during external electron scavenging systems

One set of experiments was performed to investigate the effects of scavenging electrons (hydrogen) during degradation of glucose on end product formation. Two different external electron scavenging systems were used. The first is non-biological, based on inclusion of 38 mM of thiosulfate ($S_2O_3^{2-}$) in the medium. In this part of the experiment 24.5 ml bottles were used and the L/G ratio was 0.74.

The second external electron scavenging system is biological, based on co-culture of the hydrogen producing strains with a hydrogenotrophic methanogen (HT). Here were some exceptions from the normal method of medium preparation. The 117.5 ml bottles were used. 17 ml of normal basal medium were prepared in each bottle and autoclaved. Then, the bottles were filled with gas consisting of 80% H_2 and 20% CO_2 and 1 ml of C_1, 1 ml of C_2 and 1 ml of hydrogenotrophic methanogen (Brynjarsdottir *et al.*, 2012) was added to each bottle. The bottles were incubated for 168 hours. Then the bottles are sparged and filled with nitrogen to get rid of the H_2 and the CH_4 in the gas phase. Finally, 1 ml of C_1, 0.4 ml of glucose stock solution (1M), 0.2 ml of C_2 and 0.4 ml of bacteria inoculum (J1, J2, J3 and J4 in four individual experiments) were added to the bottles. The total liquid volume of the bottles was 22 ml, corresponding to a L/G ratio of 0.23. The carbon source was 18 mM glucose and the cultures were incubated for 96 hours.

Fermentation of hydrolysates

Six different types of hydrolysates (HL) were used in this experiment; Whatman paper, newspaper, timothy grass, hemp leaf, hemp stem and straw.

The first step of the HL preparation procedure was to cut the raw materials to very small pieces with a Waring blender. For each hydrolysate, 1.67 grams of the biomass was put into three 100 ml bottles. For each hydrolysate three different pretreatments were performed. In the first bottle 66.67 ml of distilled water was added. In the second bottle, 30 ml of distilled water were added together with 0.192 ml of 97% H_2SO_4 before filling the bottle up to 66.67 ml with distilled water. The third bottle is prepared in the same way as the second bottle, except for the use of 8.33 ml of 1M NaOH instead of the acid, for a base-pretreated HL. Thus, the final concentration of each HL was 25 g/l and the concentration of acid and alkali was 0.5% (w/v) when used. The content of each bottle was mixed for a few seconds with a blender, and then autoclaved for 60 minutes.

After cooling down, the acidity of each bottle was adjusted to pH 5.0 with HCl and NaOH and 1 ml of *Novozyme* 188 and 1 ml of *Cellulast* were added to each bottle. Then the bottles were incubated in a shaking incubator at 45°C for 68 hours. After that, each HL was divided into two beakers. The acidity of one beaker was set to pH 7 and the other to pH 8. Finally, the content of each beaker was injected to a closed, sterile and nitrogen sparged 117.5 ml bottle.

Two ml of HL were used for each culture, in order to reach the initial HL concentration of 5 g/l. The 24.5 ml bottles were used and the total liquid volume of each culture was 10 ml. This corresponds to a L/G ratio of 0.69. The incubation time was 168 hours.

Total sugar and lignin analysis on timothy grass

The timothy grass that was used in the remaining experiments is described in chapter 3.4 and its composition is shown in Table 8 (Chapter 3.4). A cutting mill was used to cut down the dry grass, and sieved. Only the particle size between 0.18 mm - 1 mm was used for the experiment, but the smaller and the bigger particles were thrown away. Approximately 150 mg of the grass was balanced and put into a reaction tube. Then 1.5 ml of 72% H_2SO_4 was added to each sample. After closing the reaction tubes they are put in a shaking incubator for 60 minutes at 30°C. Then 42 ml of distilled water was added to each reaction tube and closed tightly. The tubes were autoclaved for 60 minutes and cooled down.

A few ml of the liquid fraction of each sample were filtered through a glass microbe filter (SchleicherShuell Whatman GF/C 47Ø) and transferred into HPLC vials, ready for sugar analysis.

The rest of each sample was used for the lignin analysis. Filter papers for vacuum filtration were dried in a 70°C incubator for 24 hours. Each sample was filtered through a filter paper. The filter papers with the solid material were put on petri dishes and dried at 70°C for 24 hours. At last, the filter papers were weighed and the weight gain gives the lignin content if the 150 mg sample.

Determination of total solids, volatile solids, moisture and ash in timothy grass

The biomass was dried, cut down and sieved, as described earlier. Small ceramic bowls were dried at 105°C for 17 hours, then put into a desiccator for 3 hours and then the exact weight of the ceramic bowls noted. Approximately 500 mg of biomass were put into each ceramic bowl, and the exact weight of the biomass was noted. The samples were put into a 105°C incubator for 20.5 hours, followed by one hour of desiccation. Again the exact weight was noted. Next, the samples were burned in a 575°C oven for 4 hours. The samples were weighed for the last time. By calculations, all these weight numbers can be used to analyze the total solids (TS), volatile solids (VS), moisture and ash in the timothy grass.

Determination of nitrogen in timothy grass

The nitrogen content of the timothy grass was analyzed by the Kjeldahl method. Approximately 0.5 g of biomass was weighed and put into a digestion flask. A few glass beads, one Kjeldahl catalyst tablet, 40 ml of distilled H_20 and 12.5 ml concentrated H_2SO_4 were added to each flask. The samples were digested in a digestion tower for two hours. The first hour the digestion tower was set to 180°C, but the second hour to 350°C.

The distillation process was done with 35% NaOH solution and a Kjeldahl distillation unit. The trapping flask was a 250 ml Erlenmeyer flask containing 30 ml milli-Q water, 5 ml 4% boric acid and 4-5 drops of Kjeldahl

indicator. Finally, the solution was titrated with a 0.1062 M HCl until it became colorless.

Total sugar analysis on hydrolysates of timothy grass

In this experiment, hydrolysates were made of timothy grass (described in Chapter 3.4). The procedure for the making of the HL was exactly the same as described in the chapter "3.6 - Fermentation of hydrolysates" except for two exceptions:

- Only 50 ml of each type was made instead of 66.67 ml. The concentration of all the contents was exactly the same - only the quantity was decreased.
- Instead of filtering the HL into closed and sterile bottles, at the final steps, the liquid fraction and the solid fraction were separated by filtering through a filter paper with a vacuum pump.

For all the three pretreatment methods, the samples were diluted 5 times for HPLC analysis. The concentration of the biomass was 25 g/l.

Hydrolysate fermentation by strains J1 and J4 - analyzed timothy grass

Samples of the analyzed timothy grass "Akramýri" were used for a new set of HL experiment. Whatman paper was used as a control. The procedure for the HL production was the same as described in chapter "3.6 - Fermentation of hydrolysates" except for increased quantity of this particular type of HL (only acid pretreatment (I) was used).

The setup of this HL fermentation experiment was different from the previous one. The effect of both different HL concentration and different L/G ratio was tested. To analyze the effect of different HL concentration, 2.5, 5.0 and 10.0 g/l were used. Here the 117.5 ml bottles were used. All these cultures had a total liquid volume of 60 ml, which corresponds to a L/G ratio of 1.04. To analyze the effect of different L/G ratio, 5, 30 and 45 ml of medium was used in 58 ml bottles, giving L/G ratios of 0.09, 1.07 and 3.46.

Only strains J1 and J4 were used for this experiment and the incubation time was 168 hours. This experiment was performed in triplicates.

4 Results

4.1 Screening of the University of Akureyri culture collection

In the beginning of this work all thermophilic anaerobic strains of the culture collection of the University of Akureyri were screened on glucose. Visually, growth was detected by optical density and the strains were divided into four groups; no growth, minor growth, moderate growth and major growth. A total number of 16 cultures had no growth, minor growth was observed in 13 cultures, moderate growth in 16 cultures and major growth in 80 cultures.

The 109 strains which had an observed growth were used in a second screening experiment. Each strain was cultivated with glucose, cellulose and avicel as a carbon source. With only very few exceptions, the strains did not produce end products on cellulose and avicel and then in very low concentrations. No strain was selected for further experiments based on its ability to utilize cellulose or avicel. Instead, only end product formation on glucose cultivation were used for further selection of strains.

Several strains showed good yields in producing either hydrogen or ethanol with glucose as carbon source. Table 9 shows the 20 strains which showed the best hydrogen yields and Table 10 shows the 20 best ethanol

Table 10. H_2 production and yields of the 20 best hydrogen producing strains. The production from yeast extract is substracted. The standard deviation is shown, as well as phylogenetic results from partial 16S rRNA analyzation.

Strain	H_2 production (mmol/L)	H_2 yield (mol H_2 / mol glucose)	16S rRNA analyzation
2-07-Cpa-G	34,46 ± 0,81	1,72 ± 0,04	Clostridium sp. Strain P2 (99%)
G_20_09	32,80 ± 0,36	1,64 ± 0,02	Thermoanaerobacter thermohydrosulfuricum (99.
44-07-X	25,90 ± 0,45	1,30 ± 0,02	Thermoanaerobacterium AK17 (99%)
47-07-P	23,02 ± 1,72	1,15 ± 0,09	Thermoanaerobacterium AK17 (99%)
47-07-Xo	22,92 ± 0,54	1,15 ± 0,03	–
52-07-P	22,65 ± 1,59	1,13 ± 0,08	Thermoanaerobacterium AK17 (99%)
CMC_48a_09	22,22 ± 1,71	1,11 ± 0,09	Thermoanaerobacter yonseiensis (97%)
44-07-P	22,07 ± 0,28	1,10 ± 0,01	Thermoanaerobacterium AK17 (99%)
64-07-X	21,09 ± 0,67	1,05 ± 0,03	Thermoanaerobacterium aciditolerans (98%)
9-07-X	20,85 ± 0,05	1,04 ± 0,00	Thermoanaerobacterium saccharolyticum (99%)
10-07-P	19,96 ± 1,28	1,00 ± 0,06	–
35-07-X	19,54 ± 0,48	0,98 ± 0,02	Clostridium cavendishi (98%)
21-07-Cpa-G	19,08 ± 1,21	0,95 ± 0,06	Calormator viberbiensis (97%)
G_09_09	18,09 ± 0,77	0,90 ± 0,04	–
G_04_09	17,82 ± 0,89	0,89 ± 0,04	–
54-07-P	17,53 ± 0,16	0,88 ± 0,01	Thermoanaerobacterium aciditolerans (98%)
HL/G-48	16,55 ± 1,29	0,83 ± 0,06	–
20-07-X	16,41 ± 1,66	0,82 ± 0,08	Thermoanaerobacter subcluster B (>99%)
Glu-4 c	14,87 ± 1,92	0,74 ± 0,10	Thermoanaerobacter keratinophilus (98%)
HL/G-47 c	12,50 ± 0,35	0,63 ± 0,02	Thermoanaerobacter subterraneus (99%)

Table 9. Ethanol production and yields of the 20 best ethanol producing strains. The production from yeast extract is substracted. The standard deviation is shown, as well as phylogenetic results from partial 16S rRNA analyzation.

Strain	EtOH production (mM)	EtOH yield (mol EtOH / mol glucose)	16S rRNA analyzation
20-07-Cpo	32,85 ± 0,54	1,64 ± 0,03	Thermoanaerobacter kivui (97.9%)
2-07-G	29,57 ± 1,09	1,48 ± 0,05	–
32-07-G	27,72 ± 2,32	1,39 ± 0,12	–
25-07-Cpa-G	25,85 ± 0,25	1,29 ± 0,01	Paenibacillus barengoltzii (99%)
HL/WP-47	25,22 ± 1,55	1,26 ± 0,08	Thermoanaerobacter thermohydrosulfuricus (100%
2-07-Cpa-G	25,21 ± 1,81	1,26 ± 0,09	Clostridium sp. Strain P2 (99%)
44-07-P	25,06 ± 1,97	1,25 ± 0,10	Thermoanaerobacterium AK17 (99%)
54-07-P	24,33 ± 1,66	1,22 ± 0,08	Thermoanaerobacterium acidotolerans (98%)
2-07-Cpo	23,54 ± 0,30	1,18 ± 0,02	–
44-07-X	22,49 ± 0,85	1,12 ± 0,04	Thermoanaerobacterium AK17 (99%)
21-07-Cpo	21,71 ± 1,57	1,09 ± 0,08	Thermoanaerobacter sp (<90%)
52-07-P	20,84 ± 1,46	1,04 ± 0,07	Thermoanaerobacterium AK17 (99%)
9-07-X	20,08 ± 1,68	1,00 ± 0,08	Thermoanaerobacterium saccharolyticum (99%)
47-07-Xo	18,38 ± 4,34	0,92 ± 0,22	–
HL/G-19	17,79 ± 0,60	0,89 ± 0,03	–
47-07-P	16,83 ± 2,09	0,84 ± 0,10	Thermoanaerobacterium AK17 (98%)
65-07-Xo	16,77 ± 0,94	0,84 ± 0,05	Thermoanaerobacterium saccharolyticum (100%)
24-07-X	16,63 ± 2,20	0,83 ± 0,11	Thermoanaerobacterium saccharolyticum (98%)
64-07-X	13,25 ± 1,05	0,66 ± 0,05	Thermoanaerobacterium acidotolerans (98%)
29-07-G	12,01 ± 3,16	0,60 ± 0,16	Thermoanaerobacter subcluster B (>98%)

producing strains.

Based on these results, four strains were selected for further experiments. These strains were redesignated and will hereafter be referred to as J1 (20-07-Cpo), J2 (25-07-Cpa-G), J3 (35-07-X) and J4 (G_20_09). J1 and J2 were chosen because of their high ethanol production yields and J3 and J4 because of their high hydrogen production yields.

4.2 Phylogeny

The 16S rRNA analysis revealed that the four strains chosen belong to three different genera.

The sequence from strain J1 showed that the strain is closely related to species of the genus *Thermoanaerobacter* (Figure 16). It is most closely related to *T. kivui* (97.9%), *T. sulfurigenes* (96.0%) and *T. wiegelii* (91.0%).

The sequence of strain J2 revealed that the strain belongs to the genus *Paenibacillus*. It had 99% similarity with *Paenibacillus barengoltzii*.

Strain J3 is the closest relationship to the genera *Clostridium*, according to the phylogenetic analysis (Figure 17). It is most closely related to several mesophiles, i.e. *Clostridium cavendishi* (98%), *Clostridium mesophilum* (95%), *Clostridium beijerinkii* (95%) and *Clostridium butyricum* (95%). Moreover, the strain shows 94% similarity to the moderate thermophile *Clostridium* AK14.

Like strain J1, strain J4 belongs to the genus *Thermoanaerobacter* (Figure 16). It is most closely related to *T. thermohydrosulfuricum* (99.2%), *T. brockii* (97.9%), *T. kivui* (94.8%) and *T. ethanolicus* (89.0%).

The results of the gram staining for strains J1, J2 and J4 were all gram negative. In light of difficulties with culturing strain J3 at the time the test was performed, no gram test was performed.

Microscope inspection demonstrated that all the strains are rod-shaped. Strain J4 was observed to be a bit longer and thinner than strain J1. As mentioned earlier, these strains both belong to the genus *Thermoanaerobacter*. The size and shape of strain J2 (*Paenibacillus*) is more similar to strain J1 then strain J4. The cells of strain J2 are both much shorter and thicker than observed by strain J4.

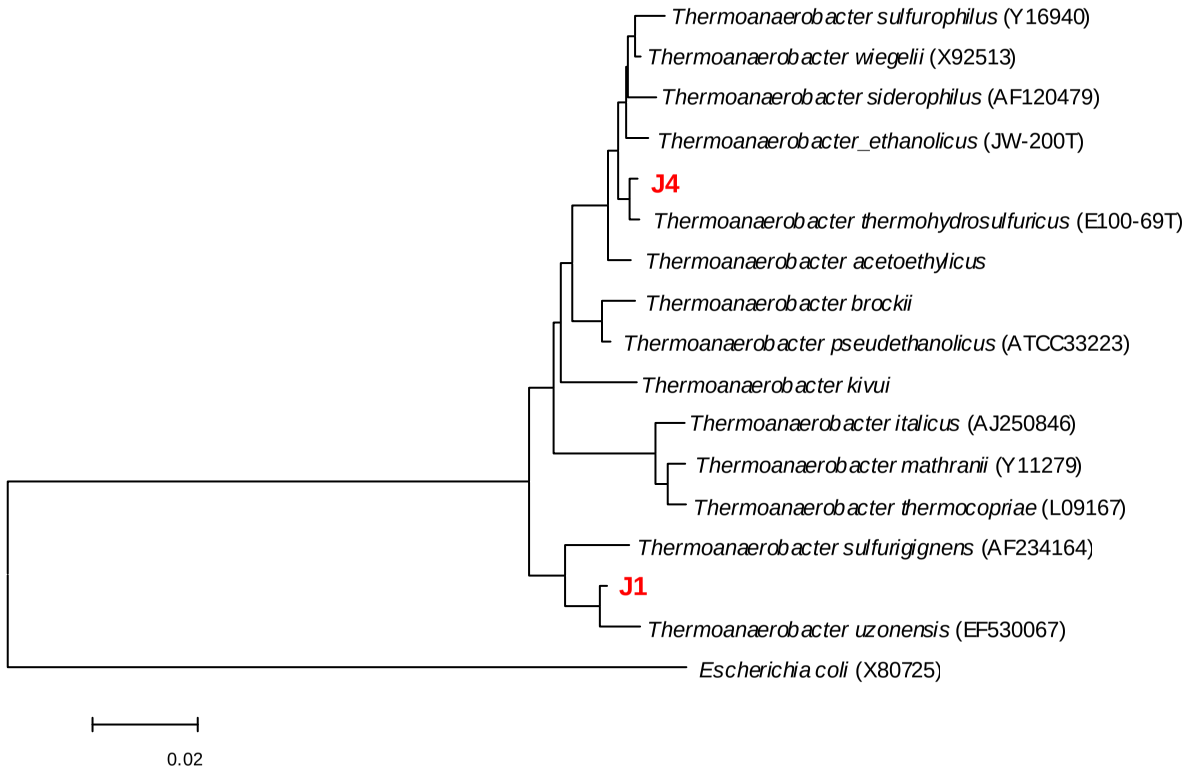

Figure 16. A phylogenetic tree for the strains with most similarity to J1 and J4 according to 16S rRNA analysis

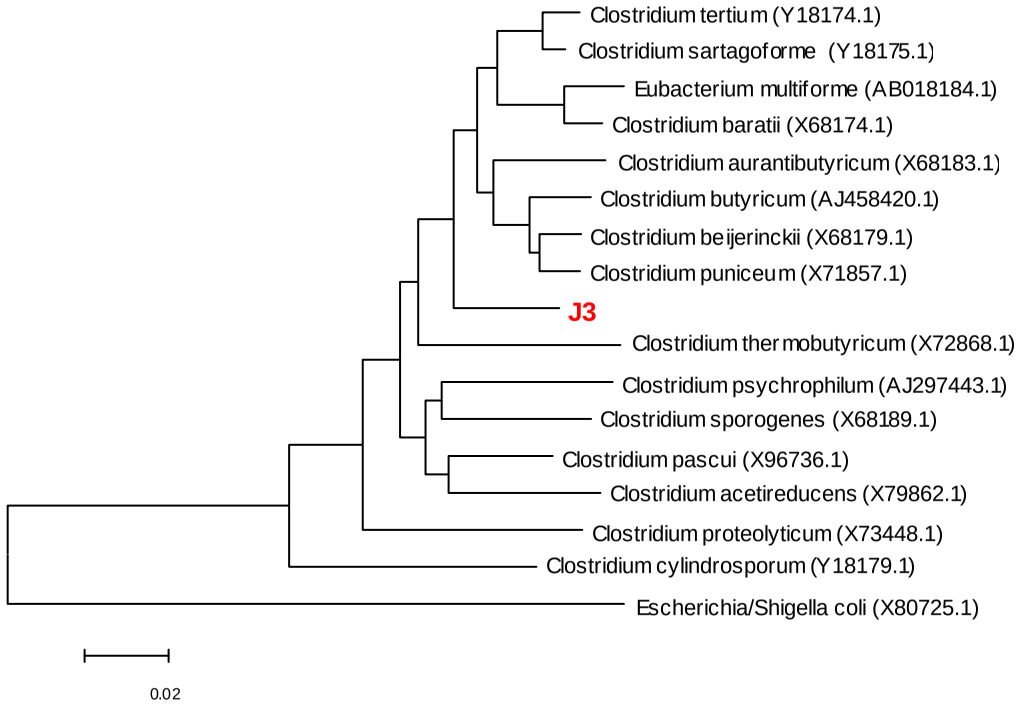

Figure 17. A phylogenetic tree for the strains with most similarity to J3 according to 16S rRNA analysis

4.3 Substrate utilization spectrum

In order to investigate the substrate utilization spectrum, each of the four strains was cultured with various carbon sources and the end products analyzed.

The results for strain J1 are displayed on Figure 18. This strain was able to utilize all the pentoses, hexoses and disaccharides that were used in this experiment, as well as the trisaccharide raffinose. The only polysaccharide J1 utilized was starch, but no utilization of cellulose, CMC, Avicel and Xylan was observed. Both pyruvate and the amino acid serine were utilized to some extent, but neither glycerol nor threonine. Ethanol was observed to be the main end product on all the carbon sources except for pyruvate and serine. Among the hexoses, the highest concentration of ethanol was observed on glucose, 38.7 mM, whereas the concentration didn't reach 30 mM on the other hexoses. Utilization of rhamnose gave the lowest ethanol yield, 20.8 mM, but by far the highest concentrations of acetate, 11.6 mM. The highest ethanol concentrations among the disaccharides were observed on sucrose, 61.2 mM. The utilization of pyruvate and serine resulted in 13.0 and 12.0 mM acetate production, respectively.

Figure 19 shows the substrate spectrum of strain J2. No utilization was observed on the pentoses xylose and arabinose, the hexoses mannose and rhamnose, the disaccharides cellobiose, sucrose and lactose. Additionally, no utilization of the polysaccharides cellulose, CMC or avicel was observed. Furthermore, no growth was observed on glycerol and trehalose. Relatively high amounts of acetate were produced on pyruvate and serine, 22.9 mM and 14.5 mM, respectively. For other carbon sources, ethanol had the highest concentration of end products. The highest ethanol concentration was observed on glucose, 28.3 mM.

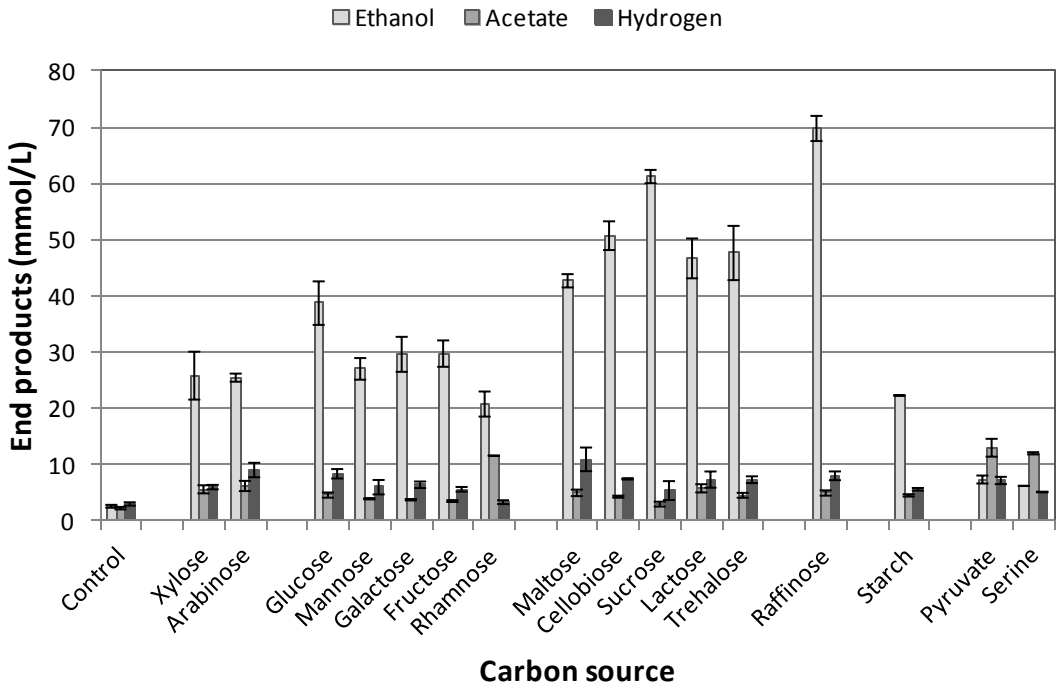

Figure 19. Substrate utilization spectrum of strain J1. End products from the carbon sources that the strain was able to utilize are shown. Error bars correspond to the standard deviation.

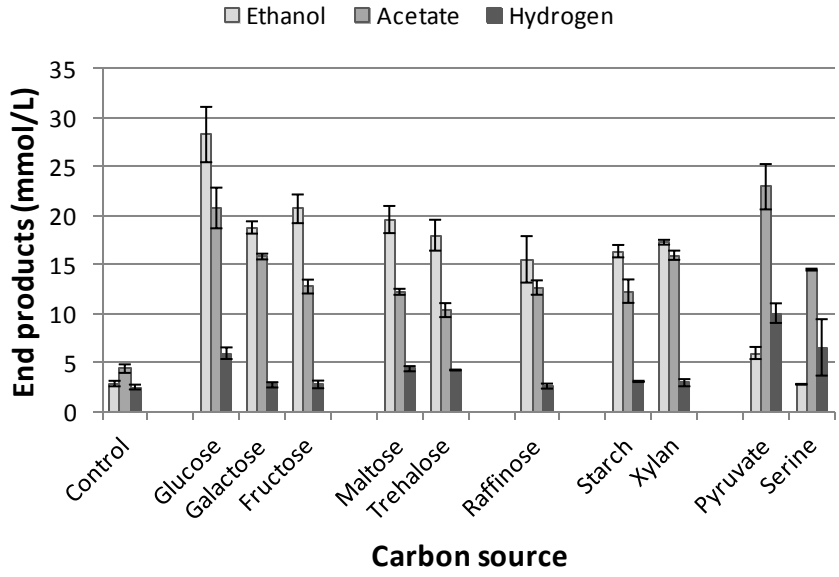

Figure 18. Substrate utilization spectrum of strain J2. End products from the carbon sources that the strain was able to utilize are shown. Error bars correspond to the standard deviation.

As shown in Figure 20, strain J3 can utilize all the hexoses and disaccharides used in the experiment, as well as the trisaccharide raffinose. On the other hand, neither pentoses nor polysaccharides were utilized. For most of the carbon sources, hydrogen, acetate and butyrate were the main end products. The highest concentrations were observed on lactose, 37.1 mmol/L hydrogen, 11.3 mM acetate and 25.0 mM butyrate. All the cultures produced from 7.8 to 11.3 mM acetate, except for rhamnose were only significant amounts of hydrogen were observed.

Figure 21 displays the results for strain J4. The sugars not observed to be utilized are arabinose, rhamnose, starch, cellulose, CMC and avicel. Furthermore, neither glycerol nor threonine showed positive results. Glucose is by far the most suitable carbon source for hydrogen production. The strain produced 36.7 mmol/L on glucose, but only 22.4 mmol/L on the second best carbon source, mannose. For most of the carbon sources utilized, hydrogen

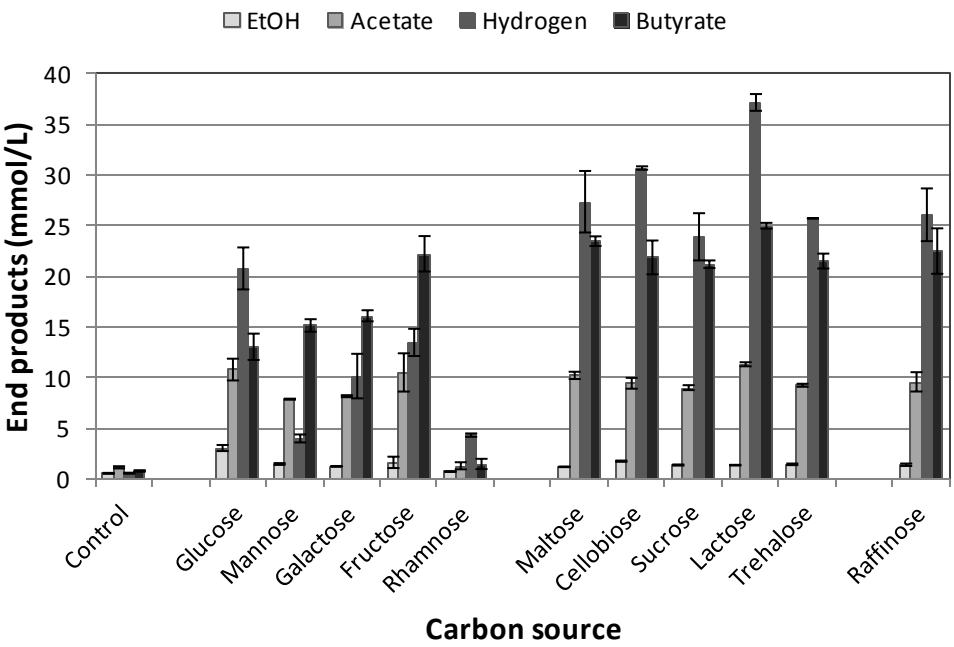

Figure 20. Substrate utilization spectrum of strain J3. End products from the carbon sources that the strain was able to utilize are shown. Error bars correspond to the standard deviation.

was the end product observed in the highest concentrations. Three of the carbon sources (cellobiose, xylan and serine) showed higher concentrations of acetate than hydrogen. On xylan, J4 produced 15.2 mM acetate and 5.1 mmol/L hydrogen. The ethanol concentrations were very low for most of the carbon sources used. The pentose xylose was an exception; 17.5 mM ethanol was produced, compared to 15.2 mM acetate and 10.7 mmol/L hydrogen.

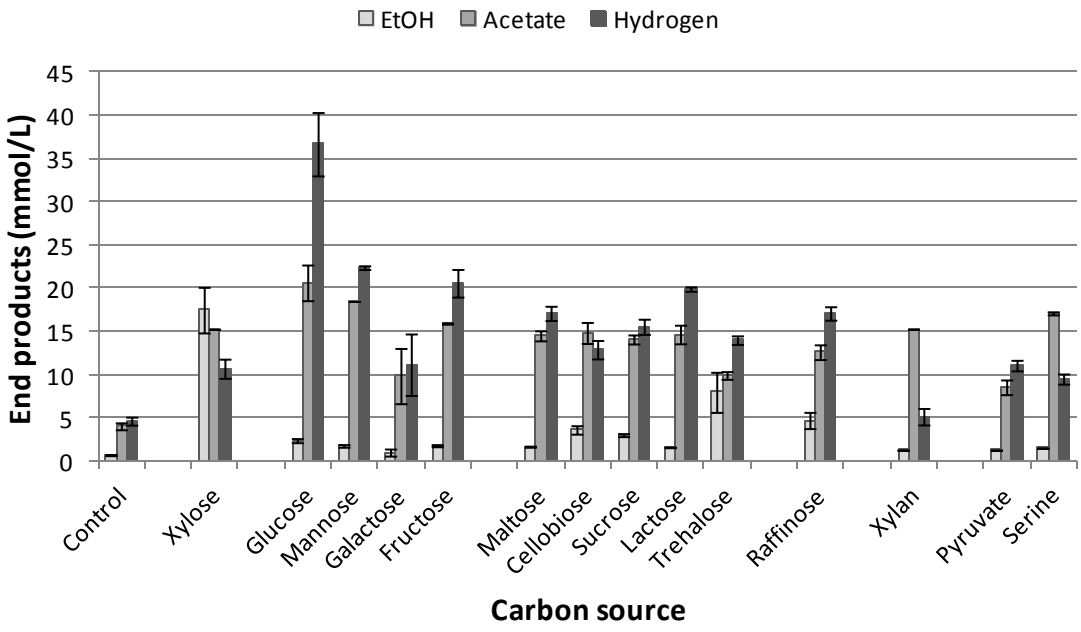

Figure 21. Substrate utilization spectrum of strain J4. End products from the carbon sources that the strain was able to utilize are shown. Error bars correspond to the standard deviation.

4.4 Experiment with different initial concentrations of glucose for strains J1, J2 and J4

This experiment was performed in order to investigate the effects of different initial glucose concentrations on its utilization and end product.

It was revealed that strain J1 did not show any sign of inhibition regarding the end product formation up to an initial concentration of 100 mM glucose (Figure 22).

The upper equation shows the approximate stochiometry of glucose (mol/mol) degradation at 10 mM initial glucose concentration, but the lower at 20 mM initial glucose concentration:

$$1.0\ \text{Glucose} \rightarrow 1.7\ \text{EtOH} + 0.4\ \text{Acetate} + 0.6\ H_2 + 2.1\ CO_2$$
$$1.0\ \text{Glucose} \rightarrow 1.7\ \text{EtOH} + 0.2\ \text{Acetate} + 0.4\ H_2 + 1.9\ CO_2$$

The ethanol concentration produced from 100 mM glucose was 120.4 mM but increased to 130.0 mM when glucose was doubled to 200 mM and glucose degradation was incomplete. Among the lower initial concentrations

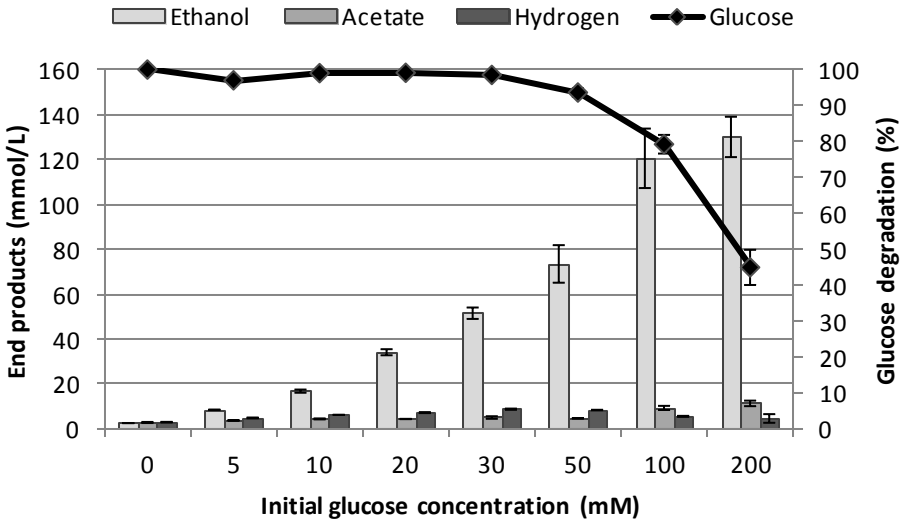

Figure 22. End product formation and proportional glucose degradation of strain J1 at different initial concentrations of glucose. Error bars correspond to the standard deviation.

of glucose, almost all the glucose was degraded. At 50 mM initial concentration of glucose, the degradation decreased to 93.6%. The degradation was 79.2% at 100 mM glucose, and 44.9% at 200 mM glucose.

Strain J2 showed signs of inhibition at much lower initial glucose concentrations than strain J1 (Figure 23). The upper equation shows the approximate stochiometry of glucose (mol/mol) degradation at 10 mM initial glucose concentration, but the lower at 20 mM initial glucose concentration:

$$1.0\ \text{Glucose} \rightarrow 1.7\ \text{EtOH} + 1.4\ \text{Acetate} + 0.7\ H_2 + 3.1\ CO_2$$
$$1.0\ \text{Glucose} \rightarrow 1.5\ \text{EtOH} + 1.0\ \text{Acetate} + 0.3\ H_2 + 2.5\ CO_2$$

At 50 mM glucose, 48.3 mM ethanol and 24.0 mM acetate were produced but increasing the glucose to 100 mM only a slight increase in ethanol was observed while the acetate produced decreased to 18.7 mM. This decrease was further established at the highest glucose concentration tested (200 mM).

Over 97% of the glucose was degraded at initial glucose concentration of 20 mM and lower. A steady drop in the glucose degradation is observed at the highest glucose concentrations; 79.4% degradation at 50 mM glucose, 38.6% at 100 mM glucose and 16.9% at 200 mM glucose.

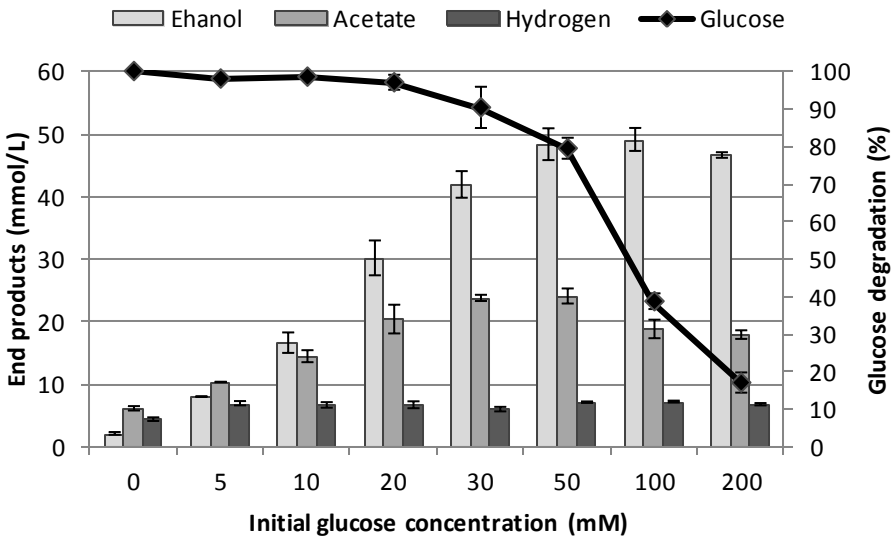

Figure 23. End product formation and proportional glucose degradation of strain J2 at different initial concentrations of glucose. Error bars correspond to the standard deviation.

As shown in Figure 24, the end product formation at different initial concentrations of glucose for strain J4 is more complex than for the other two strains. A clear inhibition is already between 20 and 30 mM initial glucose concentrations and at higher concentrations end product formation levels off and gradually less and less proportion of the glucose is degraded. A strong inhibition was observed between the glucose concentrations of 30 mM and 50 mM. The highest amounts of hydrogen are produced at 20 mM initial glucose concentration, or 22.8 mmol/L.

The acetate concentration rises in the same manner up to a glucose concentration of 20 mM, where 18.2 mM were produced. A decrease to 15.8 mM acetate is observed at 30 mM glucose, but the amount rises to 21.4 mM at the glucose concentration of 50 mM. No change in acetate production is observed at higher initial concentrations of glucose.

At the lower initial glucose concentrations (5 mM to 20 mM), less than 5 mM ethanol was produced. At 30 and 50 mM initial glucose concentrations, a dramatic increase is observed; 24.2 mM ethanol at 30 mM glucose and 34.4 mM ethanol at 50 mM glucose. The ethanol decreases to 23.1 mM at 100 mM initial concentration of glucose.

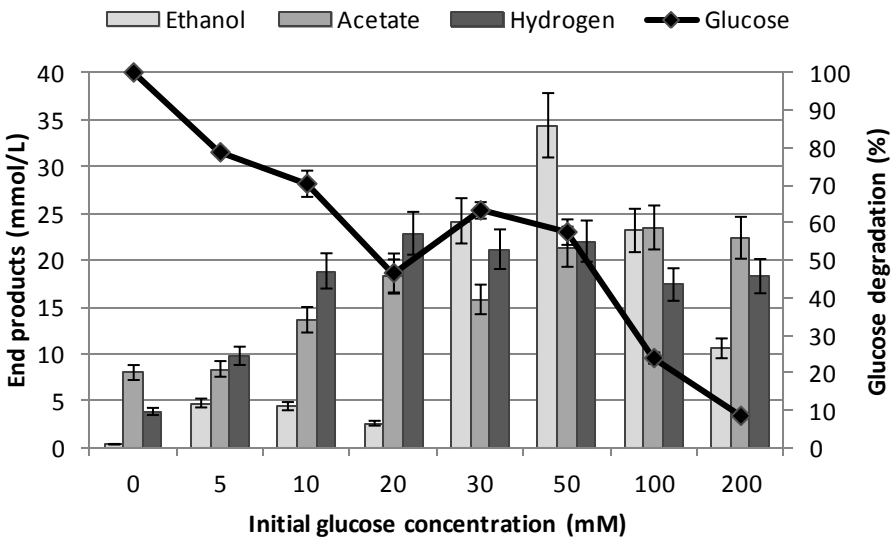

Figure 24. End product formation and proportional glucose degradation of strain J4 at different initial concentrations of glucose. Error bars correspond to the standard deviation.

Even at the lowest initial concentration of glucose used (5 mM) only 78.7% of the glucose was degraded. The proportional glucose degradation decreases in correlation with higher initial glucose concentrations, with one exception. At 20 mM glucose, 46.5% of the glucose is degraded. This increased to 63.2% at initial glucose concentration of 30 mM, in accordance with a dramatic increase in the ethanol production, as mentioned earlier. At the highest glucose concentration used (200 mM) only 8.4% of the glucose was degraded.

If the final acidity at the different initial glucose concentrations is compared to the amount of acetate produced, a clear negative correlation is observed for all the three strains (Figures 25-27). For strain J1, the amount of acetate increased by higher initial concentrations of glucose, whereas the pH decreases steadily. The greatest pH drop is observed between 50 and 100 mM initial glucose concentration; from pH 6.1 to pH 5.2. This is also when the largest increase of acetate takes place; the acetate concentration increases from 4.6 to 9.2 mM. Even though strains J2 and J4 show a few exceptions between single adjacent initial glucose concentrations, a general negative correlation between acetate production and final acidity is observed.

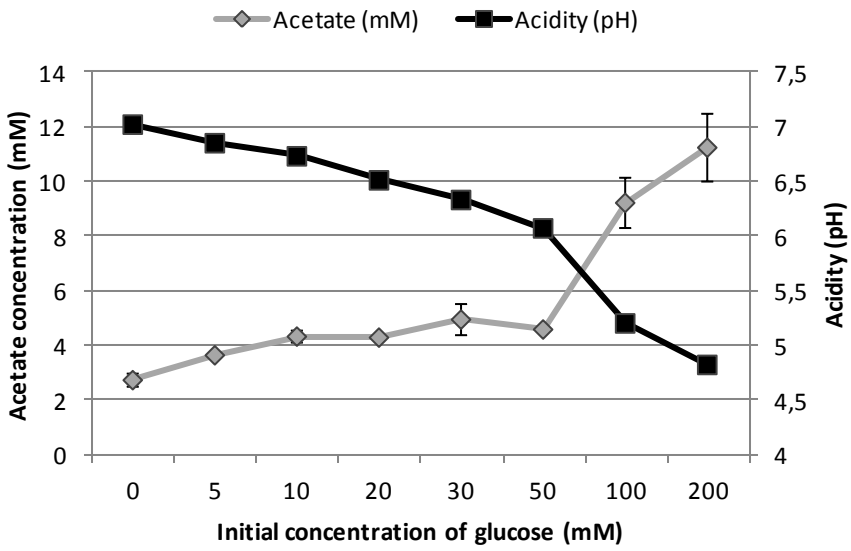

Figure 25. Acetate concentration and final acidity (pH) at different concentrations of glucose for strain J1. Error bars correspond to the standard deviation.

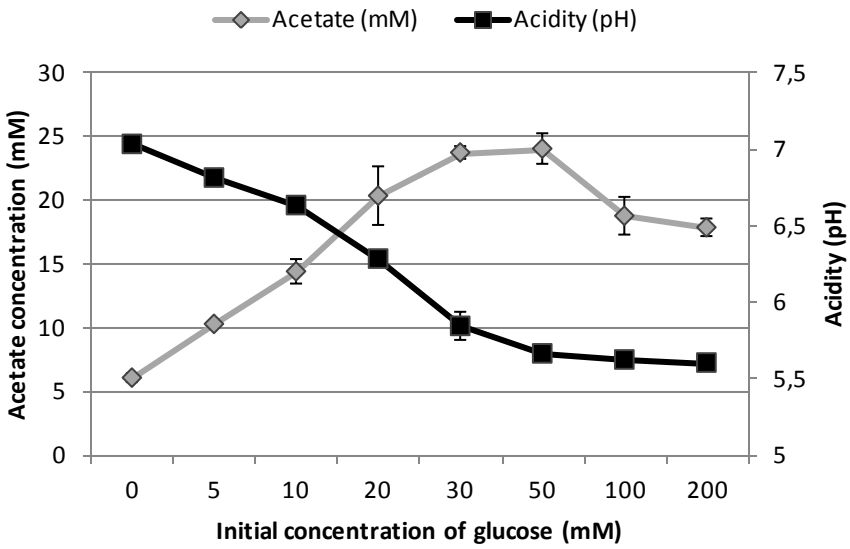

Figure 26. Acetate concentration and final acidity (pH) at different concentrations of glucose for strain J2. Error bars correspond to the standard deviation.

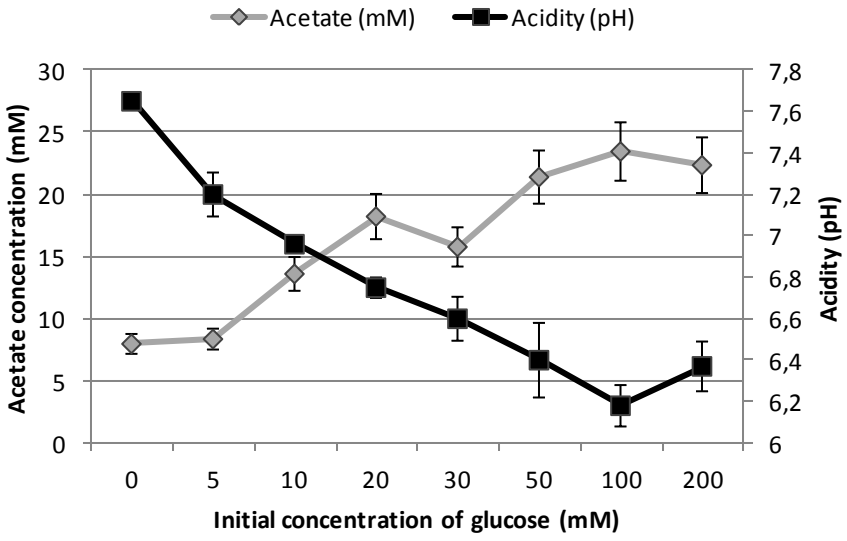

Figure 27. Acetate concentration and final acidity (pH) at different concentrations of glucose for strain J4. Error bars correspond to the standard deviation.

4.5 Effect of liquid/gas (L/G) ratio on end product formation for strains J1, J2 and J4

The influence of partial pressure of hydrogen on end product formation of strains J1, J2 and J4 was investigated by using different L/G ratios. The amount of hydrogen produced increased in accordance with higher L/G ratio for all the strains (Figure 28). The only exception was a drop at the highest L/G ratio for the most sufficient hydrogen producer, strain J4. In the following description of results, the hydrogen values are corrected to volume units with volatiles. The unit for this is millimol per liter of culture (mmol/L).

Results of end product formation for strain J1 are shown in Figure 29. The ethanol concentrations are relatively stable for all the L/G ratios used, with the exception of L/G ratio of 3.27. Ethanol concentrations of the lower L/G ratios were between 34.2 and 37.4 mM but dropped down to 30.2 mM at the highest L/G ratio used. On the other hand, acetate and hydrogen formation decreases

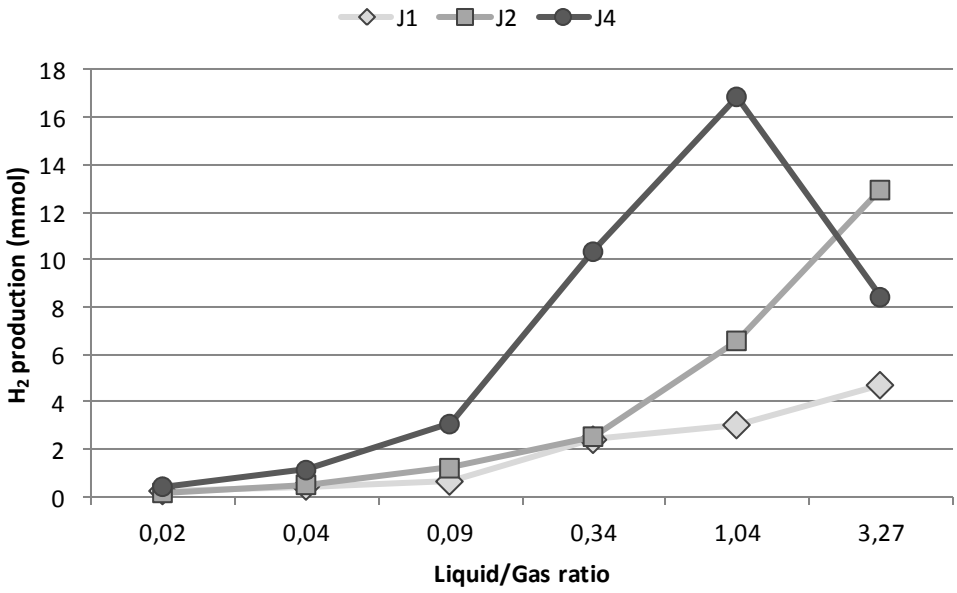

Figure 28. The amount of hydrogen produced at each L/G ratio for the strains.

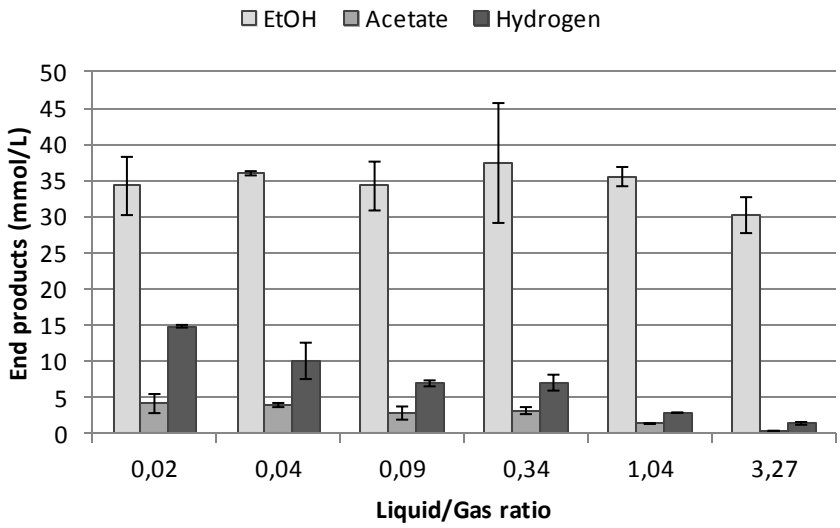

Figure 30. End product concentrations by strain J1 at various L/G ratios. The error bars correspond to the standard deviation.

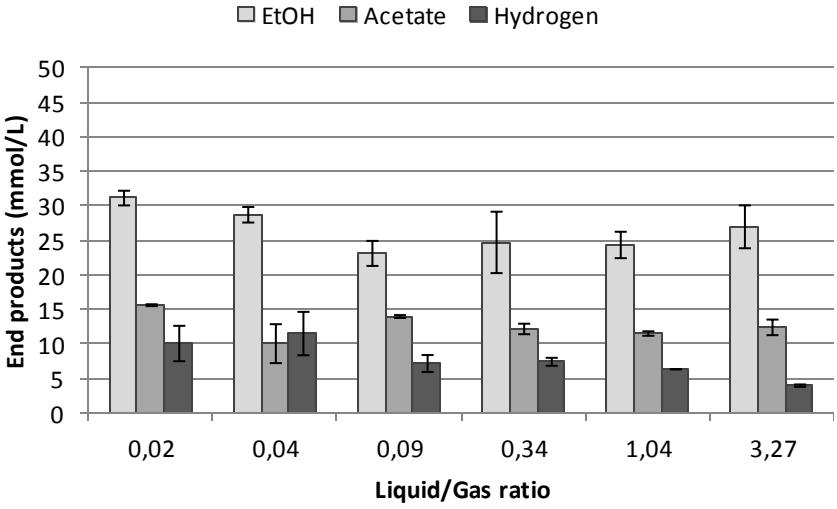

Figure 29. End product concentrations by strain J2 at various L/G ratios The error bars correspond to the standard deviation.

steadily from low to high L/G ratios. Acetate decreases from 4.2 mM to 0.4 mM and hydrogen from 14.9 to 1.4 mmol/L.

Figure 30 shows that the end product formation for strain J2 is rather stable over all the different L/G ratios. Ethanol production is highest at the lowest L/G ratio and hydrogen production decreases by more than 50% with higher L/G ratios. In all the cultures, the amount of ethanol observed is between 23.1 and 31.2 mM, and the amount of acetate between 10.0 and 15.6 mM.

Strain J4 demonstrates a shift in end product formation at different partial pressure of hydrogen (Figure 31). For the L/G ratios up to 0.34, the acetate and hydrogen production is high and stable. The highest hydrogen production was observed at the lowest L/G ratio; 49.9 mmol/L. At the L/G ratio of 1.04, a substantial decrease in the production of both acetate and hydrogen were observed whereas ethanol production increase. This was further established at the highest L/G ratio (3.27) where an almost complete shift in end product formation had occurred; only 2.4 mM acetate and 5.8 mmol/L hydrogen were produced, compared to 28.2 mM ethanol. Figure 32 shows how the hydrogen production in moles per mol glucose decreases with increased L/G ratio. The highest yield is observed at the lowest L/G ratio, 2.43 mol per mol glucose.

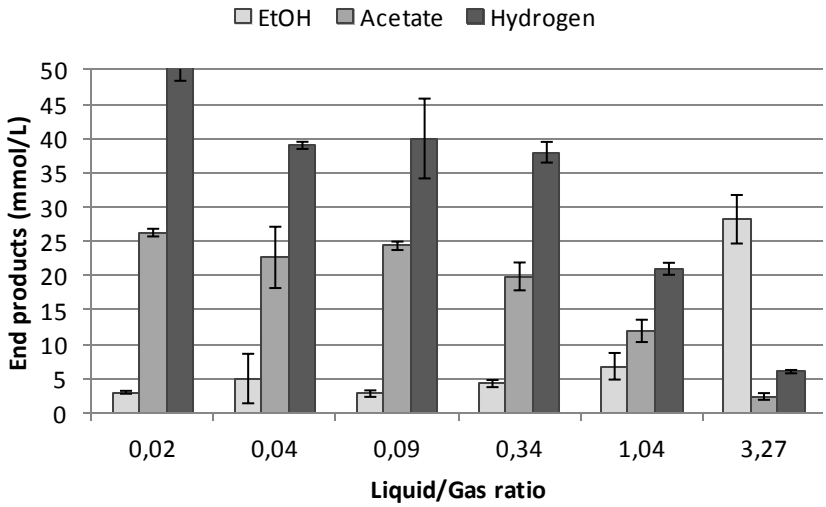

Figure 31. End product concentrations by strain J4 at various L/G ratios. The error bars correspond to the standard deviation.

Figure 33 shows the end acidity of the cultures. The acidity decreases very slowly by higher L/G ratios for strains J1 and J2. Strain J4 shows a decisive increase in pH-level from the L/G of 0.34 to 3.27. This is consistent with the dramatic shift in the end product formation, where the ethanol concentration increases rapidly opposed to a great decrease in the concentrations of acetate and hydrogen.

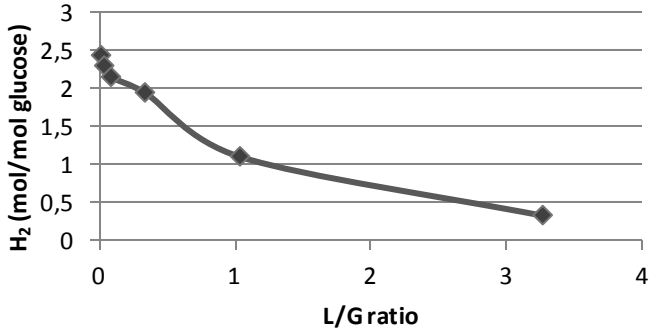

Figure 32. Hydrogen production of strain J4 in moles per mol glucose as a function of different L/G ratios.

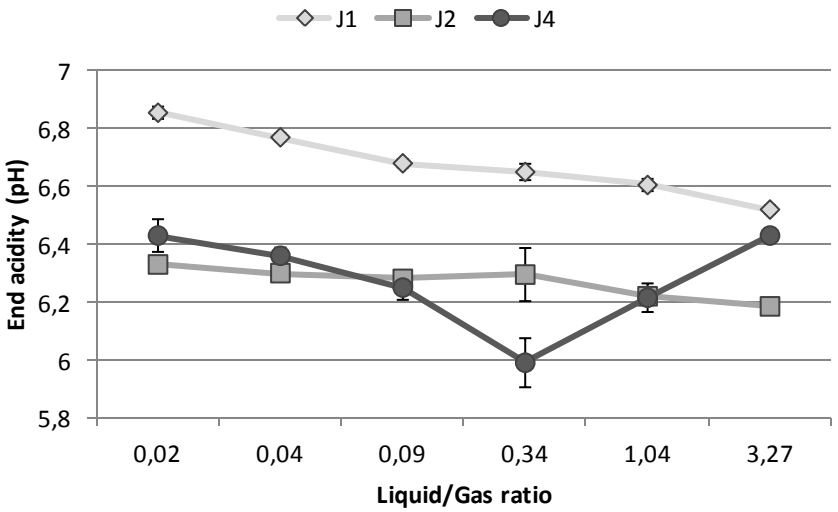

Figure 33. The end acidity (pH) at various L/G ratios for strains J1, J2 and J4. The error bars correspond to the standard deviation.

4.6 Heat resistance of strains J1, J2 and J4

An experiment was performed in order to investigate the resistance of strains J1, J2 and J4 to a 100°C heat shock over a time period from 10 up to 100 minutes. Strain J3 was not tested because of culture problems as earlier mentioned.

Interestingly, both strain J1 and J4 were able to survive the longest period of heat shock used, 100 minutes. The cultures which were derived from 100 minutes heat-shock were viable and did now show a significant decrease in end products (ethanol, acetate and hydrogen) as compared with bacteria that were not heat shocked.

On the other hand, a dramatic decrease in end product yields was observed by strain J2 between the 20 minutes and 30 minutes heat-shock period. The ethanol yield decreased from 27.4 mM to 0.8 mM, and acetate decreased from 11.8 mM to 0.8 mM. It is noteworthy that the dramatic decrease in hydrogen production is observed in the first ten minutes of heat shocking, from 20.0 mmol/L before heat shock (0 minutes) to 0,6 mmol/L at 10 minutes (Figure 34). No growth was observed in cultures that were heat-shocked for 40 minutes or more.

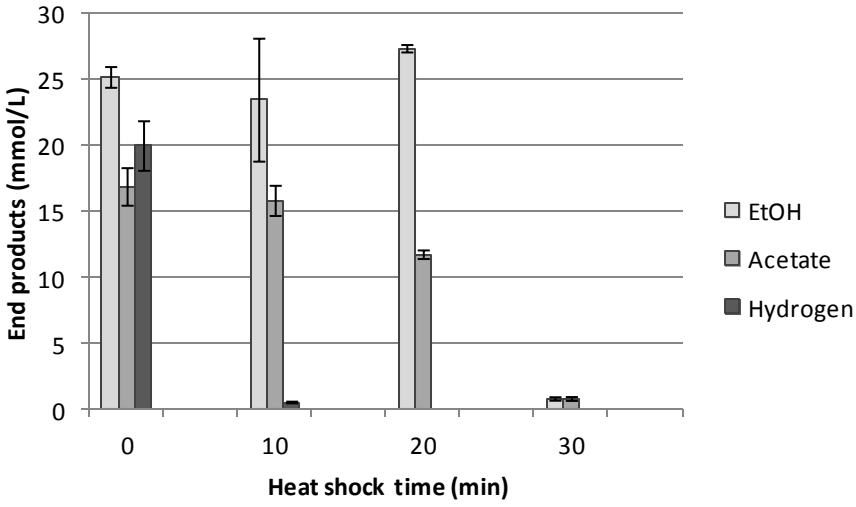

Figure 34. End product yields after different time periods of heat shocking. The error bars correspond to the standard deviation.

4.7 Ethanol tolerance of strains J1, J2 and J4

Hydrogen was used as a parameter of viable growth for the cultures when grown on different concentrations of ethanol. The observed ethanol tolerance of strain J1 was between 4.2% and 4.4% (vol/vol), as shown in Figure 35. The hydrogen production at 4,2% initial ethanol concentration was 9.4 mmol/L, but decreased to 0.8 mM at 4.4%. The hydrogen observed in the control sample, containing only yeast extract as carbon source, was 3.9 mmol/L.

The hydrogen production for strain J2 changed in a different way, compared to strain J1, when the initial concentration of ethanol was increased. Instead of decreasing dramatically at a very narrow ethanol range, the production gradually and slowly becomes less and less from 1.6% ethanol concentration, reaching less than 1.0 mmol/L at the concentration of 4.0%. Hydrogen production in the control sample was 4.0 mmol/L at 3.4% initial concentration of ethanol (Figure 36).

Similar to strain J1, strain J4 has a dramatic decrease in hydrogen production at a relatively narrow initial ethanol concentration range. As shown on Figure 37, the hydrogen concentration decreased from 12.6 mmol/L at 3.4% initial concentration of ethanol, to 4.2 mmol/L at 3.6%

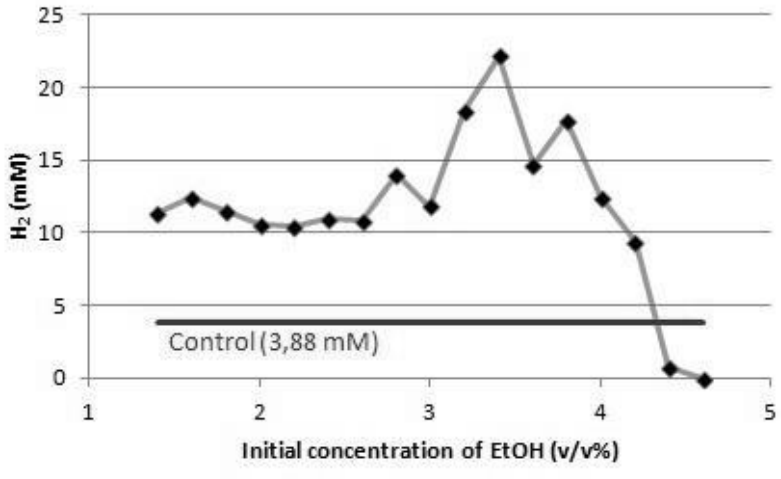

Figure 35. Hydrogen yields of strain J1 at various initial concentrations of ethanol. The red line shows the hydrogen yield of the control sample.

ethanol concentration. The control sample produced 6.0 mmol/L of hydrogen. The ethanol tolerance threshold is clearly between 3.4% and 3.6% of initial ethanol concentrations.

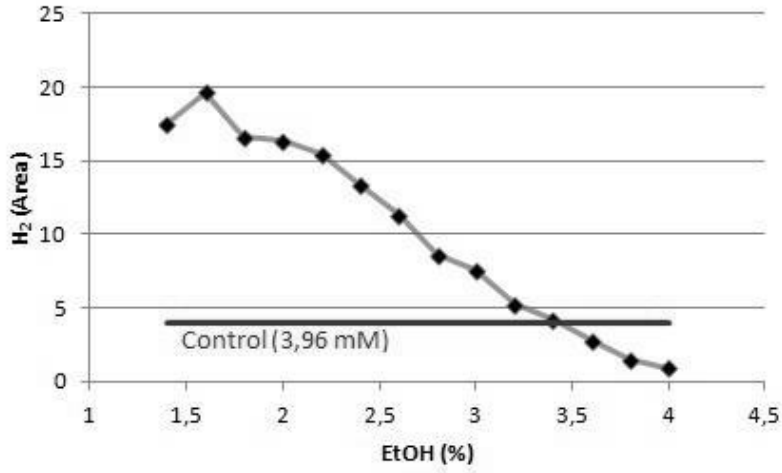

Figure 37. Hydrogen yields of strain J2 at various initial concentrations of ethanol. The red line shows the hydrogen yield of

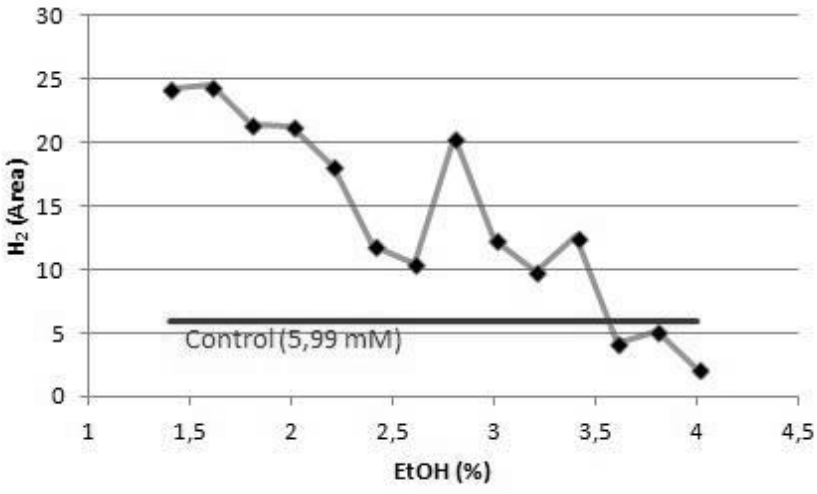

Figure 36. Hydrogen yields of strain J4 at various initial concentrations of ethanol. The red line shows the hydrogen yield of the control sample.

4.8 Fermentation during external electron scavenging systems

Two different external electron scavenging systems were used to investigate its effect on end product formation, one biological (co-culture with a hydrogenotrophic methanogen) and one non-biological (thiosulfate added to the culture). The results for strain J1 are shown in Table 11. When cultivated on glucose, the strain produced 29.0 mM ethanol, 4.2 mM acetate and 7.2 mmol/L hydrogen. Both of the electron scavenging systems were effective since almost no hydrogen was observed at the end of the experiment. However, the co-culturing with hydrogenotrophic methanogen caused a much more dramatic shift towards production of acetate (and methane), as compared to the addition of thiosulfate to the culture. The biological electron scavenging system reduced the ethanol concentration to 4.1 mM and increased acetate to 29.5 mM and 7.4 mmol/L methane was produced. Thiosulfate only reduced the ethanol concentration to 20.0 mM and increased

Table 11. End product formation of strain J1 in the presence of thiosulfate or a hydrogenotrophic methanogen. Standard deviation is shown.

	Concentration (mmol/L)			
	Ethanol	**Acetate**	**Hydrogen**	**Methane**
Control	3.0 ± 0.1	2.9 ± 0.1	2.0 ± 0.1	0.0 ± 0.0
Control + S_2O_3	1.1 ± 0.1	5.2 ± 0.2	0.3 ± 0.0	0.0 ± 0.0
Control + methanogen	0.9 ± 0.5	4.9 ± 0.4	0.0 ± 0.0	2.4 ± 0.0
Glucose	29.0 ± 1.5	4.2 ± 0.3	7.2 ± 0.5	0.0 ± 0.0
Glucose + S_2O_3	20.0 ± 0.3	15.5 ± 2.1	0.3 ± 0.0	0.0 ± 0.0
Glucose + methanogen	4.1 ± 0.2	29.5 ± 1.2	0.5 ± 0.0	7.4 ± 1.2

Table 12. End product formation of strain J2 in the presence of thiosulfate or a hydrogenotrophic methanogen. Standard deviation is shown.

	Concentration (mmol/L)			
	Ethanol	**Acetate**	**Hydrogen**	**Methane**
Control	1.9 ± 0.2	1.3 ± 0.3	0.2 ± 0.0	0.0 ± 0.0
Control + S_2O_3	1.7 ± 0.1	1.3 ± 0.1	0.1 ± 0.0	0.0 ± 0.0
Control + methanogen	1.5 ± 0.1	1.3 ± 0.2	0.0 ± 0.0	0.2 ± 0.0
Glucose	25.1 ± 0.7	12.8 ± 0.4	5.9 ± 0.0	0.0 ± 0.0
Glucose + S_2O_3	27.7 ± 0.5	14.0 ± 0.7	0.0 ± 0.0	0.0 ± 0.0
Glucose + methanogen	24.4 ± 1.0	16.3 ± 0.6	0.0 ± 0.0	0.6 ± 0.1

acetate to 15.5 mM.

The electron scavenging systems had almost no effect on the end product formation of strain J2 (Table 12). 5.9 mmol/L hydrogen were observed when the strain was cultured on glucose without the scavenging systems. The amounts of acetate rise slightly using the electron scavenging systems and 0.6 mM methane was produced when using the biological electron scavenging system.

Table 13 shows the results for strain J3. The thiosulfate culture was not successful since observed amounts of hydrogen did increase instead of decreasing as expected. On the other hand, the hydrogenotrophic methanogen used nearly all the hydrogen in the co-culture and produced 6.5 mmol/L methane. A slight decrease in ethanol concentration (1.3 mM to 1.1 mM) and a slight increase in acetate concentration (10.4 mM to 10.6 mM) was observed.

Table 14. End product formation of strain J3 in the presence of thiosulfate or a hydrogenotrophic methanogen. Standard deviation is shown.

	Concentration (mmol/L)				
	Ethanol	**Acetate**	**Hydrogen**	**Methane**	**Butyrate**
Control	0.4 ± 0.0	1.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.2
Control + S_2O_3	0.4 ± 0.0	1.0 ± 0.1	0.9 ± 0.1	0.0 ± 0.0	1.2 ± 0.1
Control + methanogen	0.8 ± 0.1	1.5 ± 0.3	0.1 ± 0.0	1.3 ± 0.3	1.3 ± 0.1
Glucose	1.3 ± 0.1	10.4 ± 1.9	18.5 ± 1.2	0.0 ± 0.0	12.2 ± 0.3
Glucose + S_2O_3	1.5 ± 0.0	9.0 ± 0.1	22.6 ± 0.9	0.0 ± 0.0	12.4 ± 0.3
Glucose + methanogen	1.1 ± 0.0	10.6 ± 0.8	0.2 ± 0.0	6.5 ± 0.2	10.9 ± 1.3

Table 13. End product formation of strain J4 in the presence of thiosulfate or a hydrogenotrophic methanogen. Standard deviation is shown.

	Concentration (mmol/L)			
	Ethanol	**Acetate**	**Hydrogen**	**Methane**
Control	1.5 ± 0.0	3.8 ± 0.5	6.1 ± 0.5	0.0 ± 0.0
Control + S_2O_3	1.5 ± 0.2	4.3 ± 0.6	1.8 ± 0.1	0.0 ± 0.0
Control + methanogen	2.2 ± 0.2	3.6 ± 0.2	0.2 ± 0.0	2.8 ± 0.3
Glucose	2.2 ± 0.0	20.6 ± 1.2	35.8 ± 2.2	0.0 ± 0.0
Glucose + S_2O_3	2.0 ± 0.2	23.7 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
Glucose + methanogen	1.5 ± 0.0	36.1 ± 0.3	0.0 ± 0.0	16.2 ± 1.4

The biological electron scavenging system does also seem to have more effect on the end product formation of strain J4 than the thiosulfate (Table 14). Whereas the presence of thiosulfate decreased the ethanol production slightly from 2.2 mM to 2.0 mM, the hydrogenotrophic methanogen was able to push the produced amounts down to 1.5 mM. More dramatically, the inclusion of the methanogen raised acetate production from 20.6 mM to 36.1 mM, but thiosulfate raised the amounts up to 23.7 mM.

4.9 Fermentation of hydrolysates

All four strains were cultivated on six different types of hydrolysates which were pretreated in three different ways.

The end product formation for strain J1 is shown in Figure 38. In most

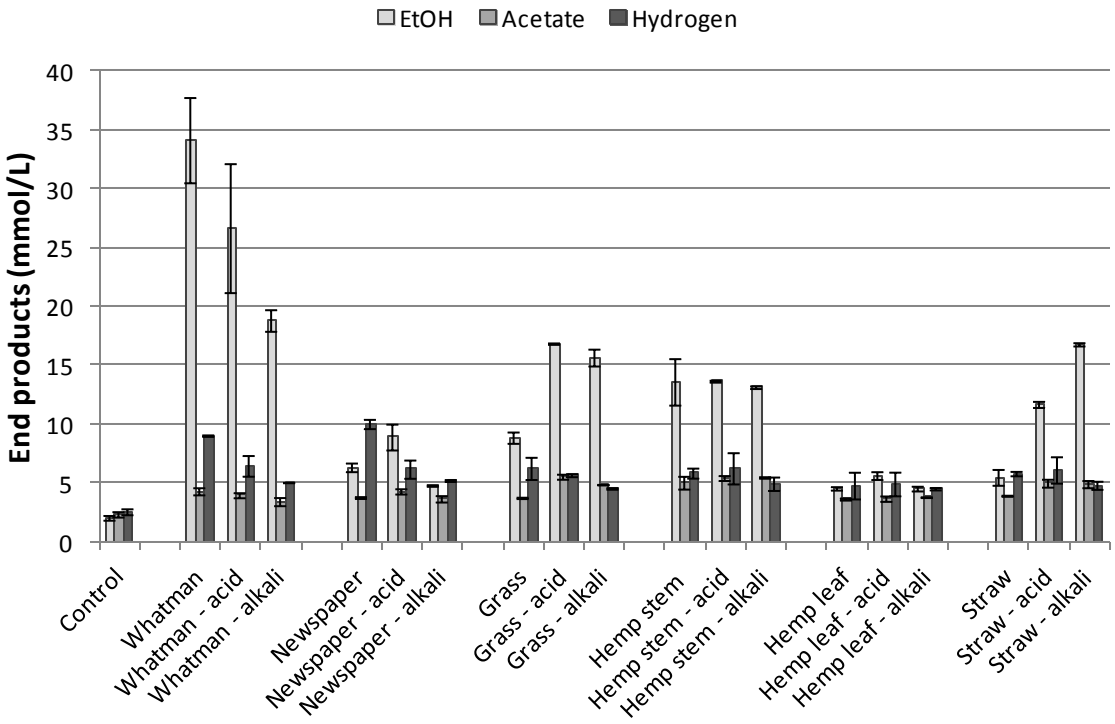

Figure 38. End product formation for strain J1 on various types of hydrolysates, pretreated in three different ways. The first set of columns for each hydrolysate shows results for unpretreated hydrolysates, the second one shows acid pretreated and the third one alkali pretreated. The error bars correspond to the standard deviation.

of the cultures, ethanol was the main end product. The highest concentrations of ethanol were produced on Whatman paper where 34.1 mM were produced on the unpretreated hydrolysate, 26.7 mM on the acid pretreated and 18.8 mM on the alkali pretreated. On hemp stem, 13.1 to 13.6 mM ethanol were produced, with hardly any significant difference between the three pretreatment methods. On grass and straw, pretreatment with acid and alkali increase the ethanol production significantly. The ethanol concentrations on unpretreated hydrolysates were 8.8 mM for grass and 5.5 mM for straw. When the hydrolysates were pretreated with acid, these amounts rose up to 16.8 mM for grass and 11.7 mM for straw. Increased concentrations of ethanol were observed when pretreated with alkali as well; where concentrations were 15.7 mM for grass and 16.8 mM for straw. On hemp leaf and newspaper the ethanol concentrations did not reach 10 mM for any of the pretreatments. Only two of the cultures had hydrogen production over 6.5 mmol/L. The concentration of hydrogen on the unpretreated Whatman paper was 9.0 mmol/L, and 10.0 mmol/L on the unpretreated newspaper. Pretreatments with both acid and alkali reduced the hydrogen production on both of these hydrolysates.

As displayed in Figure 39, the end product formation for strain J2 is more complicated than for strain J1. No specific end product were dominant for all the different hydrolysates but the effect of different pretreatment was more significant. The highest ethanol concentrations are observed on Whatman paper. The unpretreated sample and the one pretreated with acid, resulted in ethanol concentrations of 23.6 mM and 23.3 mM, respectively. When pretreated with alkali, the ethanol concentration dropped to 17.5 mM. If compared to the unpretreated sample, the hydrogen production increased most dramatically in the alkali pretreated sample; from 5.1 mmol/L to 15.3 mmol/L.

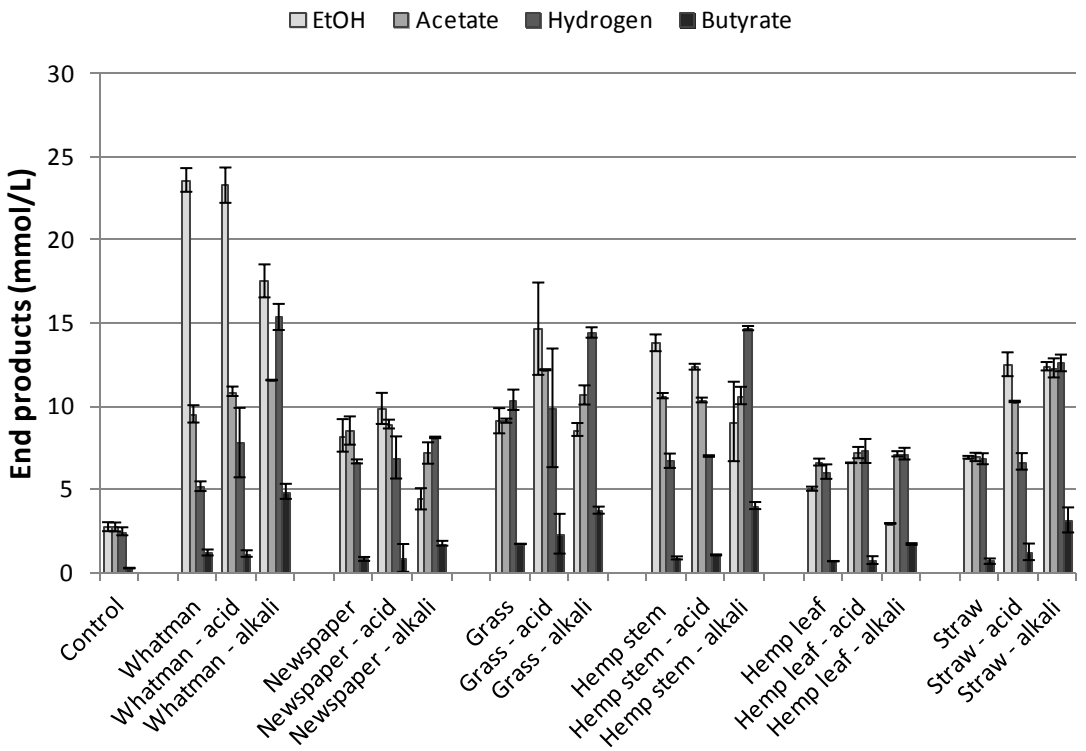

Figure 39. End product formation for strain J2 on various types of hydrolysates, pretreated in three different ways. The first set of columns for each hydrolysate shows results for unpretreated hydrolysates, the second one shows acid pretreated and the third one alkali pretreated. The error bars correspond to the standard deviation.

The alkali pretreatment seemed to have great impact on both hemp stem and straw hydrolysates. In the case of hemp stem, the ethanol dropped from 13.8 mM to 9.0 mM if compared to the unpretreated hydrolysate. Instead, the hydrogen observed increased from 6.7 mmol/L to 14.7 mmol/L, and the butyrate from 0.8 mM to 4.0 mM. On the other hand, all the four end products increased when the straw hydrolysate was pretreated with alkali. The ethanol concentration increased from 6.9 mM to 12.4 mM, acetate from 6.9 mM to 12.3 mM and hydrogen from 6.8 mmol/L to 12.6 mmol/L. The amount of butyrate rose from 0.6 mM to 3.1 mM.

Strain J3 was in-effective in fermenting different types of hydrolysates, as revealed in Figure 40. The only type of hydrolysate where a certain end

product reached 8 mmol/L was Whatman paper. Acetate production on Whatman paper was almost the same for all the three pretreatment types; between 7.0 mM and 8.0 mM. The highest hydrogen production was observed when no pretreatment was performed, or 27.5 mmol/L, whereas lower values, 24.1 mmol/L and 17.2 mmol/L, were observed when pretreated with acid and alkali, respectively. Chemical pretreatment with acid and alkali on Whatman paper seem to have negative effects on end product formation of strain J3.

J3 was the only butyrate producer of the four strains studied. On Whatman paper, J3 produces 17.8 mM butyrate without pretreatment, and

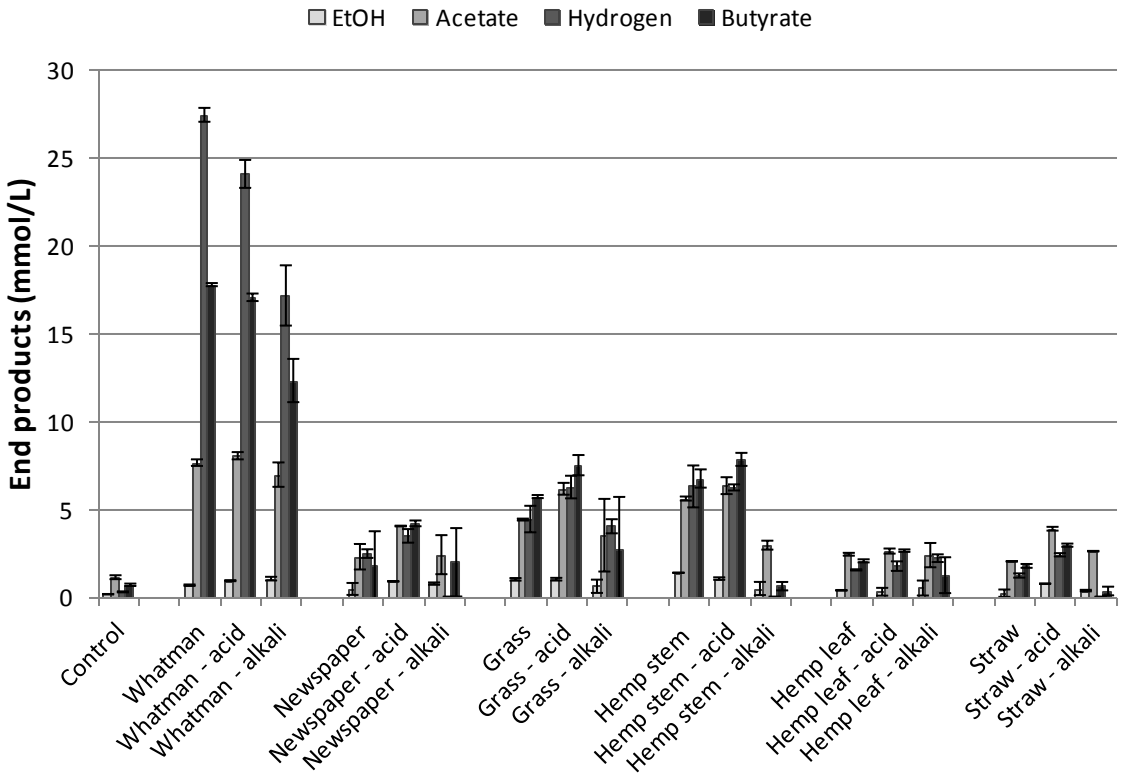

Figure 40. End product formation for strain J3 on various types of hydrolysates, pretreated in three different ways. The first set of columns for each hydrolysate shows results for unpretreated hydrolysates, the second one shows acid pretreated and the third one alkali pretreated. The error bars correspond to the standard deviation.

17.1 mM when pretreated with acid. On grass and hemp stem, butyrate was the end product that was observed in the highest concentrations, both without pretreatment and when pretreated with acid. For the acid pretreatment, J3 produced 7.5 mM butyrate on grass and 7.8 mM on hemp stem.

Figure 41 shows the end product formation for strain J4. The strain produced mainly acetate and hydrogen from all the different types of hydrolysates used. The highest concentrations observed were on unpretreated Whatman paper; 14.9 mM acetate and 25.5 mmol/L

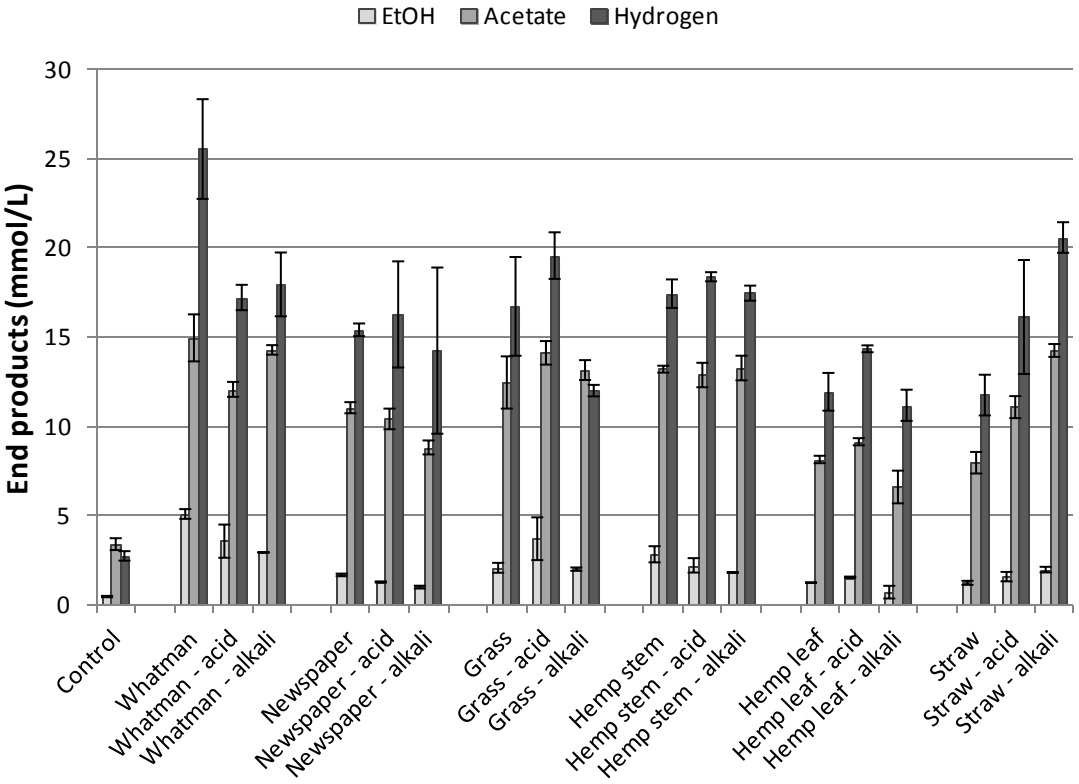

Figure 41. End product formation for strain J4 on various types of hydrolysates, pretreated in three different ways. The first set of columns for each hydrolysate shows results for unpretreated hydrolysates, the second one shows acid pretreated and the third one alkali pretreated. The error bars correspond to the standard deviation.

hydrogen. When J4 was cultured on straw, a pretreatment with both acid and alkali increase the amounts of acetate and hydrogen produced. The acetate concentrations observed were 7.9 mM when no pretreatment was used, 11.9 mM when pretreated with acid and 14.2 when pretreated with alkali. 11.7 mmol/L hydrogen were produced in the unpretreated sample, 16.1 mmol/L when pretreated with acid and 20.5 mmol/L when pretreated with alkali.

4.10 Chemical analysis of Timothy grass

Earlier (Chapter 4.09), the capacity of the strains to utilize several different types of hydrolysates was investigated and the end product formation analyzed. From the results obtained it was decided to investigate two strains, one good ethanol producer (J1) and one good hydrogen producer (J4) in more detail on grass hydrolysate to characterize their biofuel production potential from lignocellulosic biomass. Additionally, a full chemical analysis was done on the grass used which was not done in earlier experiments.

Two samples of Timothy grass, called Akramýri and Tjarnarspilda, were analyzed. The composition of the main chemical content of the grass is shown in Table 8 in Chapter 3.4.

The sugars glucose, xylose and arabinose, along with lignin, nitrogen, ash and moisture, account for over 88% of the biomass weight in both of the two samples (Table 15).

The total solids and the volatile solids of the grass from Akramýri was 92.2% and 86.6%, respectively, giving the moisture content to be 8.8%. Similar values were observed for the grass from Tjarnarspilda; total solids, volatile solids and moisture were 92.0%, 85.6% and 8.0%, respectively.

Total sugar and lignin analysis in solid samples revealed that approximately 36% to 37% of the grass weight were glucose and about 15% xylose whereas arabinose was much less. The lignin content of both samples

Table 15. Weight proportion composition of the Timothy grass samples Akramýra and Tjarnarspilda. Standard deviation is shown.

Content	Weight proportion (% wt/wt)	
	Akramýri	Tjarnarspilda
Lignin	17.09 ± 1.74	18.42 ± 0.95
Glucose	37.43 ± 1.26	35.66 ± 0.99
Xylose	15.27 ± 1.53	15.17 ± 1.26
Arabinose	4.13 ± 1.13	4.32 ± 1.50
Nitrogen	1.26 ± 0.03	1.05 ± 0.13
Ash	4.29 ± 0.11	5.39 ± 0.22
Moisture	8.78 ± 0.17	8.03 ± 0.11
Total	88,25	88,04

were similar, about 17% to 18%.

In order to analyze the sugar composition of hydrolysates derived from Timothy grass, hydrolysates were made from these two samples. The same pretreatment methods were used as earlier.

Acid pretreatment was found to be most effective pretreatment method for both of the grass types (Table 16 and Table 17). More than 43 mM glucose and 51.4 mM xylose was observed for acid pretreatment for Akramýri grass (biomass concentration of 25 g/L) , but 42.0 mM glucose and 55.5 mM xylose for Tjarnarspilda grass samples.

Table 16. Sugar contents of the Timothy grass sample Akramýri with different pretreatment methods. Proportion of each sugar in the hydrolysates, compared to total sugar analysis, is shown. The amount of sugar observed is displayed as well ± standard deviation.

	Glucose		Xylose		Arabinose	
Pretreatment method	Concentration (mM)	Proportion in HL (%)	Concentration (mM)	Proportion in HL (%)	Concentration (mM)	Proportion in HL (%)
No pretreatment	38.7 ± 1.2	74.4	40.7 ± 0.8	159.9	0	0
Acid pretreatment	43.4 ± 0.1	83.5	51.4 ± 0.3	202.0	5.5 ± 0.6	80.3
Alkali preatment	40.6 ± 0.6	78.1	32.4 ± 0.5	128.2	3.3 ± 0.1	47.6

Table 17. Sugar contents of the Timothy grass sample Tjarnarspilda with different pretreatment methods. Proportion of each sugar in the hydrolysates, compared to total sugar analysis, is shown. The amount of sugar observed is displayed as well ± standard deviation.

	Glucose		Xylose		Arabinose	
Pretreatment method	Concentration (mM)	Proportion in HL (%)	Concentration (mM)	Proportion in HL (%)	Concentration (mM)	Proportion in HL (%)
No pretreatment	39.8 ± 2.2	80.3	49.1 ± 2.3	194.1	0	0
Acid pretreatment	42.0 ± 2.9	84.7	55.5 ± 3.3	219.5	5.6 ± 0.6	77.9
Alkali preatment	38.0 ± 0.3	76.7	32.4 ± 0.5	128.2	0	0

4.11 Hydrolysate fermentation by strains J1 and J4 - analyzed Timothy grass

After full analysis of the timothy grass a second fermentation experiment was performed. Only "Akramýri" timothy grass was investigated, using Whatman paper as a control. Two types of experiments were done: different HL concentration (2.5, 5.0 and 10.0 g/L) and different L/G ratios (0.09, 1.07 and 3.46).

Less than 3 mM glucose were left in the culture broth of strain J1 during fermentation of the Whatman paper and grass HL regardless of different initial concentration (Table 18). The ethanol production increased from 18.0 mM at 2.5 g/L HL concentration to 67.7 mM at 10 g/L from Whatman paper HL, whereas acetate and hydrogen were produced in minor and similar amounts at all concentrations. Sugars were not analyzed by HPLC at the end of fermentation time, only the total concentration of sugars was determined using the anthrone method. The concentration of ethanol produced from grass HL were 11.7 mM at 2.5 g/L but increased to 41.8 mM at 10.0 g/L. Acetate production increased from 4.7 mM at 2.5 g/L to 8.1 mM at 10 g/L but the hydrogen production was similar at all concentrations used. Different L/G ratios were used with a constant HL concentration of 5.0 g/L (Table 19). Again, strain J1 degraded almost all the sugars present in both the timothy grass and Whatman paper HLs. The different L/G ratios did not affect the ethanol production of the strain, which was constant between 36.2 mM and 40.3 mM in the Whatman paper HL and between 25.5 mM and 25.8 mM in the grass HL. For acetate and hydrogen production, negative correlation was between L/G ratios and amounts produced per liquid volume.

Strain J4 utilized much less of the glucose present in the Whatman paper HL as compared with strain J1. In the lowest concentration used, only 54% of the glucose was degraded and this decreased to 31.7% and 19.0% at 5.0 and 10.0 g/L, respectively (Table 20). For grass HL, especially at 5 and 10 g/L, also the majority of sugars were not degraded. The main end products of this strain, acetate and hydrogen, did not increase in a proportional with the increased initial concentrations as observed with ethanol production by strain J1. The strain produced between 12.0 and 14.2 mM acetate in different concentration of Whatman paper HL and 10.9 and 18.1 mmol/L hydrogen whereas values for the grass HL were 9.0 to 12.5 and 10.8 to 12.1 mmol/L,

respectively. In the experiment with different L/G ratios on Whatman paper HL, about one third of the glucose was degraded at higher L/G ratios but almost 60% at the lowest L/G ratio used (Table 21). The results from the grass HL show more efficient degradation of sugars at low L/G ratio. Also, a fundamental shift in end product formation is observed between the different L/G ratios. The highest ethanol production is observed at the highest L/G ratio (10.0 mM), whereas acetate and hydrogen concentrations are low (6.9 mM and 5.3 mmol/L, respectively). When the L/G ratio is decreased from 3.46 to 1.07, the acetate concentration nearly doubles (to 12.5 mM) and the hydrogen concentration increases to 12.3 mmol/L. On the other hand, the ethanol production decreases to 3.1 mM. The shift is more dramatic when the L/G ratio is decreased further, to 0.09. The production of acetate and hydrogen increases to 21.8 mM and 38.0 mmol/L, respectively, whereas the concentration of ethanol decreases to 2.7 mM.

Table 18. Results of hydrolysate experiment for strain J1 on analyzed timothy grass with different initial hydrolysate concentrations.

Hydrolysate (HL)	HL conc. (g/L)	Initial concentrations			Final sugar concentr. (mM)	End products (mmol/L)			Final acidity (pH)
		Glucose (mM)	Xylose (mM)	Arabinose (mM)		Ethanol	Acetate	Hydrogen	
Control	0.0	NA	NA	NA	NA	2.3 ± 0.1	2.4 ± 0.1	2.9 ± 0.4	6.94 ± 0.01
Whatman paper	2.5	15.4*	NA	NA	2.8 ± 0.5	18.0 ± 1.5	4.1 ± 0.3	6.4 ± 1.7	6.57 ± 0.03
Whatman paper	5.0	30.9*	NA	NA	2.1 ± 0.3	35.5 ± 2.8	4.1 ± 0.5	4.6 ± 0.9	6.25 ± 0.04
Whatman paper	10.0	61.7*	NA	NA	2.5 ± 0.3	67.7 ± 3.4	2.6 ± 0.1	4.8 ± 0.3	5.35 ± 0.05
Timothy grass	2.5	4.3	5.1	0.6	2.6 ± 0.2	11.7 ± 0.6	4.7 ± 0.3	4.0 ± 0.2	6.70 ± 0.01
Timothy grass	5.0	8.7	10.3	1.1	1.0 ± 0.2	21.1 ± 0.6	5.6 ± 0.2	3.4 ± 0.2	6.49 ± 0.02
Timothy grass	10.0	17.4	20.6	2.2	0.8 ± 0.1	41.8 ± 1.8	8.1 ± 0.2	3.9 ± 0.6	6.07 ± 0.08

* Calculated theoretical values

Table 19. Results of hydrolysate experiment for strain J1 on analyzed timothy grass with different L/G ratios.

Hydrolysate (HL)	L/G ratio	Initial concentrations			Final sugar concentr. (mM)	End products (mmol/L)			Final acidity (pH)
		Glucose (mM)	Xylose (mM)	Arabinose (mM)		Ethanol	Acetate	Hydrogen	
Control	0.09	NA	NA	NA	NA	1.9 ± 0.1	5.7 ± 0.2	6.7 ± 0.5	7.27 ± 0.01
Control	1.07	NA	NA	NA	NA	1.8 ± 0.2	2.7 ± 0.1	2.5 ± 0.3	6.97 ± 0.01
Control	3.46	NA	NA	NA	NA	1.7 ± 0.1	2.3 ± 0.1	1.7 ± 0.2	6.94 ± 0.00
Whatman paper	0.09	30.9	NA	NA	1.6 ± 0.1	40.1 ± 1.8	8.4 ± 0.1	15.2 ± 1.0	6.49 ± 0.02
Whatman paper	1.07	30.9	NA	NA	2.2 ± 0.1	37.4 ± 0.6	4.5 ± 0.2	4.9 ± 0.4	6.20 ± 0.01
Whatman paper	3.46	30.9	NA	NA	2.4 ± 0.2	36.2 ± 0.7	4.7 ± 1.3	2.7 ± 0.4	6.16 ± 0.05
Timothy grass	0.09	8.7	10.3	1.1	1.6 ± 0.2	25.8 ± 1.2	9.6 ± 0.4	11.1 ± 0.7	6.47 ± 0.04
Timothy grass	1.07	8.7	10.3	1.1	2.1 ± 0.1	25.5 ± 5.3	6.0 ± 0.5	3.3 ± 0.2	6.49 ± 0.01
Timothy grass	3.46	8.7	10.3	1.1	2.6 ± 0.0	25.6 ± 1.8	4.8 ± 0.4	1.9 ± 0.1	6.44 ± 0.01

* Calculated theoretical values

Tafla 20. Results of hydrolysate experiment for strain J4 on analyzed timothy grass with different initial hydrolysate concentrations.

Hydrolysate (HL)	HL conc. (g/L)	Initial concentrations			Final sugar concentr. (mM)	End products (mmol/L)			Final acidity (pH)
		Glucose (mM)	Xylose (mM)	Arabinose (mM)		Ethanol	Acetate	Hydrogen	
Control	0.0	NA	NA	NA	NA	0.3 ± 0.0	3.1 ± 0.1	5.0 ± 0.1	7.72 ± 0.01
Whatman paper	2.5	15.4*	NA	NA	7.4 ± 0.5	1.7 ± 0.5	12.0 ± 1.5	18.1 ± 2.7	6.94 ± 0.03
Whatman paper	5.0	30.9*	NA	NA	21.1 ± 1.6	2.2 ± 0.5	13.1 ± 1.2	16.1 ± 0.6	6.53 ± 0.06
Whatman paper	10.0	61.7*	NA	NA	50.0 ± 3.0	7.8 ± 5.7	14.2 ± 0.5	10.9 ± 1.9	5.38 ± 0.59
Timothy grass	2.5	4.3	5.1	0.6	2.5 ±0.4	1.4 ± 0.2	9.0 ± 1.0	12.1 ± 1.5	7.19 ± 0.05
Timothy grass	5.0	8.7	10.3	1.1	11.0 ±1.3	3.6 ± 0.8	9.6 ± 1.0	11.9 ± 2.1	7.03 ± 0.06
Timothy grass	10.0	17.4	20.6	2.2	27.8 ±1.9	5.4 ± 0.2	12.5 ± 0.5	10.8 ± 1.5	6.65 ± 0.08

* Calculated theoretical values

Table 21. Results of hydrolysate experiment for strain J4 on analyzed timothy grass with different L/G ratios.

Hydrolysate (HL)	L/G ratio	Initial concentrations			Final sugar concentr. (mM)	End products (mmol/L)			Final acidity (pH)
		Glucose (mM)	Xylose (mM)	Arabinose (mM)		Ethanol	Acetate	Hydrogen	
Control	0.09	NA	NA	NA	NA	0.5 ± 0.1	5.1 ± 0.4	7.3 ± 0.8	7.82 ± 0.02
Control	1.07	NA	NA	NA	NA	0.3 ± 0.0	3.1 ± 0.3	3.6 ± 0.4	7.70 ± 0.01
Control	3.46	NA	NA	NA	NA	0.2 ± 0.0	2.4 ± 0.2	2.4 ± 0.1	7.73 ± 0.04
Whatman paper	0.09	30.9	NA	NA	12.4 ± 0.8	3.5 ± 0.7	23.5 ± 0.6	41.3 ± 6.0	6.46 ± 0.02
Whatman paper	1.07	30.9	NA	NA	18.4 ± 1.4	2.8 ± 0.1	9.7 ± 0.6	12.3 ± 0.5	6.41 ± 0.03
Whatman paper	3.46	30.9	NA	NA	18.2 ± 0.7	20.3 ± 2.3	5.9 ± 1.3	4.8 ± 0.5	6.78 ± 0.08
Timothy grass	0.09	8.7	10.3	1.1	4.4 ± 0.3	2.7 ± 0.5	21.8 ± 0.4	38.0 ± 3.4	6.46 ± 0.05
Timothy grass	1.07	8.7	10.3	1.1	15.1 ± 0.5	3.1 ± 1.5	12.5 ± 1.8	12.3 ± 1.3	6.87 ± 0.07
Timothy grass	3.46	8.7	10.3	1.1	14.2 ± 1.1	10.0 ± 1.4	6.9 ± 0.6	5.3 ± 0.6	6.94 ± 0.12

* Calculated theoretical values

5 Discussion

5.1 Phylogeny and substrate spectrum

The present study is on four strains of thermophilic bacteria isolated from hot springs in Iceland. Initially, all strains presently available in the culture collection of the University of Akureyri were screened on glucose to classify them as either good ethanol or hydrogen producers. This resulted in the selection of these four strains, where two were classified as good ethanol producers (J1 and J2) and two as good hydrogen producers (J3 and J4). Two of the strains belong to the genus *Thermoanaerobacter* (J1 and J4), one *Paeniobacillus* (J2) and one *Clostridium* (J3). The *Thermoanaerobacter* strains are "true" thermophiles with temperature optima above 65°C (results not shown) whereas the other strains are moderate thermophiles with temperature optima around 50°C.

All the strains were subjected to various experiments to investigate their suitability for biofuel production; for example by studying the substrate spectrum, the effects of partial pressure of hydrogen, the effect of different substrate loadings and the tolerance to heat shock and ethanol concentrations. However, strain J3 was extremely difficult to grow after several months and repeated experiments to reactivate it from older cultures (both from freeze and from room temperature) were unsuccessful. Thus, data on several environmental factors were not included for this strain in all cases.

Phylogenetically, strain J1 and J4 belong to the genus of *Thermoanaerobacter*. According to the Euzeby list of procaryotes, the genus currently consist of 18 species. Strain J1 is most closely related to *T. kivui* (97.9%), *T. sulfurigenes* (96.0%) and *T. wiegelii* (91.0%) based on full 16S sDNA sequencing. Strain J4 is most closely related to *T. thermosulfuricum* (99.2%), *T. brockii* (97.9%), *T. kivui* (94.8%) and *T. ethanolicus* (89.0%). The genus *Thermoanaerobacter* falls into Clusters V in the phylogenetic interrelationship of *Clostridium* according to Collins and co-workers (Collins, *et al.*, 1994). The taxonomy of the genus was refined by Lee and co-

workers (Lee *et al.*, 1993) but since then many new species have been described, renamed (e.g. *T. brockii*), or reassigned to a new genus such as *Caldanaerobacter*. All *Thermoanaerobacter* species are obligate anaerobes degrading a broad spectrum of various carbohydrates as well as amino acids producing a mixture of ethanol, acetate, lactate, hydrogen and carbon dioxide (Cayol *et al.*, 1995; Cook *et al.*, 1996; Lee *et al.*, 1993; Lee *et al.*, 2007; Wiegel & Ljungdahl, 1981).

Strain J1 degraded all the 13 carbohydrates tested, as well as pyruvate and serine. Strain J4 also has a broad substrate spectrum, degrading 11 of the 13 carbohydrates tested, and also both pyruvate and serine. When comparing these results with other *Thermoanaerobacter* strains, their substrate spectrum is similar or broader than the type strain *Thermoanaerobacter ethanolicus* which does not degrade cellobiose, raffinose and rhamnose (Wiegel & Ljungdahl, 1981). Moreover, Thermoanaerobacter wiegelii does neither degrade arabinose nor rhamnose (Cook *et al.*, 1996). End product formation from carbohydrates were mostly ethanol (J1) and acetate (J4) together with hydrogen and carbon dioxide (Figures 18 and 21 in Chapter 4.3). Both strains were capable of using thiosulfate as electron acceptors as reported for most *Thermoanaerobacter* species (Fardeau *et al.*, 1996; Fardeau *et al.*, 1997). No species within the genus has been reported to degrade cellulose which is in accordance with the results presented in this thesis. Both strains have also been shown to be able to degrade the branched chain amino acids in presence of either thiosulfate or a hydrogenotrophic methanogen (results not shown) but this has also been shown by *Thermoanaerobacter brockii* (Fardeau, *et al.*, 1996).

Strain J2 is a good ethanol producer (producing more than 1.5 mol ethanol per mol glucose at initial glucose concentration of 20 mM and L/G ratio 1.04) and belongs to the genus *Paenibacillus*. This is a huge genus, containing 144 species according to the Euzeby list of procaryotes and exist in many kinds of habitats, e.g. plant materials, soil and from geothermal areas (Ash *et al.*, 1993; Logan *et al.*, 2004; Khianngam *et al.*, 2009; Behrendt *et al.*, 2010; Zhou *et al.*, 2012). Most bacteria within this genus are facultative aerobes and of mesophilic origin (Lal *et al.*, 2012; Baek *et al.*, 2010; Stieglmeier *et al.*, 2009). Several moderate thermophiles have been described, such as *Paenibacillus thermophilus*, *Paenibacillus thermoaerophilus*, *Paenibacillus marinum* and *Paenibacillus tezpurensis*

(Zhou *et al.*, 2012; Ueda *et al.*, 2013; Bouraoui *et al.*, 2013; Rai *et al.*, 2010). The substrate spectrum of J2 was quite broad; degrading 10 of 13 carbohydrates tested. The strain did not show any hydrolytic activity on any of the polymers used, nor on serine or pyruvate.

Strain J3 is most closely related to the genus *Clostridium* Cluster IV. The strain was most closely related to several mesophiles, i.e. *Clostridium cavendishi* (98%), *Clostridium mesophilum* (95%), *Clostridium beijerinkii* (95%) and *Clostridium butyricum* (95%). The strain also shows 94% similarity to the moderate thermophile *Clostridium* AK14, still not valid species which was isolated from the same geothermal area as strain J3 (Almarsdottir *et al.*, 2010). Strain AK14 is also a good hydrogen producer with classical acetate-butyrate fermentation pattern as strain J3. Both strains have a relatively narrow growth range of only about 15°C, ranging approximately from 40°C to 55°C (results not shown). This could be caused by the ecological niche of their origin, but the temperature of the hot springs both strains were isolated from was around 50°C.

5.2 Ethanol and hydrogen production from sugars

The interest in thermophilic ethanol and hydrogen producing bacteria has rapidly increased recently due to concerns regarding the sustainable production of fuels. Ethanol production among thermophiles has been known for a long time and its interest peaked after the oil crisis in the 1970's. This inspired the isolation of several well-known bacteria within the genus *Thermoanaerobacter*, such as *T. brockii* and *T. ethanolicus* (Wiegel & Ljungdahl, 1981; Cayol *et al.*, 1995; Lee *et al.*, 1993). *T. ethanolicus* has been reported to show the highest ethanol yields of among thermophilic anaerobic bacteria, or 1.9 mol ethanol per mol glucose which is close to the theoretical maximum (2 mol ethanol per mol glucose). Several other thermophiles have been shown to have yields between 1.5 and 1.8 mol ethanol per mol hexose (Table 2).

Strain J1 shows very good ethanol yields, or 1.7 mol ethanol per mol glucose and 1.25 mol ethanol per mol xylose, which corresponds to theoretical yields of 85% and 75%, respectively. Strain J2 is also a very good ethanol producer with 1.5 mol ethanol per mol glucose, but the strain did not

utilize xylose. Lal and co-workers showed that *Paenibacillus polymyxa* is able to produce at least 0.5 mol ethanol per mol glucose (Lal *et al*., 2012). Moreover, the genus has been reported to produce ethanol from glycerol substrate (Gupta *et al*., 2009), but J3 did not utilize glycerol.

It is a well-described phenomenon that temperature is of importance for hydrogen production by bacteria due to thermodynamics. At lower temperatures, bacteria are unable to produce hydrogen from NADH because the ΔG for the biochemical reactions becomes unfavorable. This results in the production of reduced end products such as ethanol and lactate, leading to lower hydrogen yields. The maximum hydrogen yields may be obtained if hexoses are degraded to only one type of volatile end product, acetate, according to the following stoichiometry:

$$1\,\text{Glucose} \rightarrow 2\,\text{Acetate} + 2\,CO_2 + 4\,H_2$$

This is, however, never accomplished unless at very high temperatures. Thus, extremophiles such as *Caldicellulosiruptor* and *Thermotoga* are known as the best hydrogen producers among thermophilic bacteria. However, bacteria that belong to the genera of *Thermoanaerobacterium*, *Thermoanaerobacter* and *Clostridium* have also been reported to have reasonable high hydrogen yields from carbohydrates (Sigurbjornsdottir & Orlygsson, 2012; Cao *et al*. 2009; Pattra *et al.*, 2008; Soboh *et al*., 2009; Saripan & Reungsang, 2013; Khamtib & Reungsang, 2012). *Thermoanaerobacter tengcongensis* has been reported to produce 4 moles of hydrogen from 1 mol of glucose (100% theoretical yields) during the active removal of hydrogen from the culture (Soboh *et al*., 2004). *Thermoanaerobacter* strain J4 is a good hydrogen producer and produced 36.7 mmol/L and 10.7 mmol/L on glucose and xylose (20 mM), respectively. This corresponds to almost 2 mol hydrogen per mol glucose but glucose was not completely degraded in this case and higher yields were reported during other culture conditions (see later). Table 4 shows some of the best hydrogen producing thermoanaerobes in comparison with strain J3 and J4.

5.3 The effect of different initial glucose concentrations on end product formation

An important environmental factor concerning the utilization of thermophilic bacteria for scale up production of ethanol and hydrogen is the substrate loading. It is a well-known fact that thermophilic bacteria do not tolerate high substrate loadings compared to yeasts and fungi (Lacis & Lawford, 1992; Sommer *et al.*, 2004). The main reason for this can be caused by increasing osmotic stress by increasing substrate loadings or by end product inhibition, either directly due to acid formation or indirectly as a result of low pH caused by acid formation (Olsson & Hahn-Hagerdal, 1996; van Ginkel & Logan, 2005).

Of the three strains tested in present investigation, strain J1 was the most tolerant strain concerning increased glucose loadings in batch cultures. The strain degraded all glucose up to 50 mM and more than 80% at 100 mM glucose concentrations. Strain J2 was also was tolerant up to 30 mM initial glucose concentrations, but strain J4 was very sensitive at lower concentrations. The most reasonable explanation for the insensitivity of strain J1 is that the inhibition is caused by low pH rather than by the sugar itself, but due to low acetate production the pH decreases less than by the other strains.

5.4 The effect of partial pressure of hydrogen on end product formation

Another important environmental factor that influences the end product formation spectra of thermophilic bacteria is the partial pressure of hydrogen (pH_2) (Ben-Bassat *et al.*, 1981; Abreu *et al.*, 2012; Hawkes *et al.*, 2002). It is well-established that elevated hydrogen production in batch cultures inhibits hydrogenases and directs the electron flow to more reduced end products like ethanol, butyrate, lactate, and even alanine (Levin *et al.*, 2004; Nath & Das, 2004). To investigate this in detail it was decided to cultivate three of the strains in large (117.5 mL) serum bottles with different L/G ratios. Thus, the

L/G ratio was lowest 0.017 and highest 3.27. Again a striking difference was observed by the high ethanol producing strains (J1 and J2) compared to the acetate/H_2 producing strain, J4 (this experiment was not done on strain J3). Both J1 and J2 produced high amounts of ethanol under all pH_2 tested. Strain J1 produced between 30.2 and 37.4 mM ethanol (highest at L/G ratio 0.34) and strain J2 between 24.4 and 31.1 mM ethanol (highest at L/G ratio 0.017). Strain J4, however, produced most acetate and hydrogen at the lowest L/G used, or 26.3 mM acetate and 25.8 mmol/L hydrogen. At the highest L/G used, these values had decreased dramatically; 2.4 mM acetate and 2.6 mmol/L hydrogen were produced, whereas a dramatic increase in ethanol production was observed (28.2 mM). This is in good correlation with other high acetate/hydrogen producing strains (Almarsdottir *et al.*, 2010; Orlygsson & Baldursson, 2007; Ciranna *et al.*, 2012).

5.5 Ethanol and heat tolerance

One of the main drawbacks for the use of thermophilic bacteria for ethanol production is their low tolerance towards ethanol. Most wild type thermophilic bacteria only tolerate 2-3% (vol/vol) ethanol but the highest tolerance reported was a mutant strain of *Thermoanaerobacter ethanolicus*, capable of tolerating up to 11.4% (Carreira *et al.*, 1983). One strain that was developed from this strain (JW200) however showed much less tolerance (Hild *et al.*, 2003). Another *Thermoanaerobacter* strain BG1L1 and *Thermoanaerobacter thermohydrosulfuricus* have been shown to tolerate between 10.1% and 10.5% (vol/vol) ethanol in continuous culture studies (Georgieva *et al.*, 2008; Ng *et al.*, 1981). By transferring the parent strain (39E) to successively higher concentrations of ethanol, an alcohol tolerant strain (39A) was obtained (Lovitt *et al.*, 1988). The mutant strain grows at over 10% ethanol concentrations at 45°C but only to 4.2% at 68°C. Ethanol yields decreased from 1.5 mol ethanol per mol glucose to 0.6 mol ethanol per mol glucose. Present investigation showed that strains J1, J2 and J4 were completely inhibited between 3.2% and 4.4% ethanol which is in good correlation with most thermophilic anaerobes (Georgieva *et al.*, 2007b; Tsai *et al.*, 2011).

The two "true" thermophiles (J1 and J4) were extremely heat resistant, tolerating more than 100 minutes at 100°C. Some of *Thermoanaerobacter*

strains, such as *T. brockii*, *T. wiegelii*, *T. thermohydrosulfuricus* and *T. pentosaceus* (Cayol *et al.* 1995; Cook *et al.*, 1996; Balk *et al.*, 2009, Tomas *et al.*, 2012) have been observed to form spores. A spore staining test was performed for strains J1, J2 and J4, but it was not successful. However, it is very likely that strains J1 and J4 form spores since they are able to survive boiling temperatures for such a long period. The moderate thermophile tested, J2, only tolerated 100°C for several minutes which is not surprising because of the lower temperature optimum observed.

5.6 Electron scavenging systems

The fact that two of the strains investigated in present study belong to the genus *Thermoanaerobacter* was of interest due to the fact that these bacteria can reduce oxidized sulfur compounds like thiosulfate (S_2O_3). The manipulation of the electrons produced during oxidation of carbon substrates is of importance concerning energy gain and deeper understanding of the physiology of the strains. Another way to scavenge electrons during substrate oxidation is to co-culture the strains with a hydrogenotrophic methanogen. Thus, both biological and non-biological electrons scavenging possibilities are possible and were used in present study.

Strain J1, which is highly ethanolgenic, produced about 1.3 mol ethanol per mol glucose and 0.11 mol acetate per mol glucose without any electron acceptor. These yields changed to 0.85 and 0.63 with thiosulfate and to 0.05 and 13.3 in co-culture of hydrogenotrophic methanogen. Thus, the strain was strongly affected by electron removal and changing its end product formation towards more oxidized products (acetate). This is a little surprising since when this strain was cultivated with different L/G ratios the different pH_2 did not change end product formation. Thus, there seem to be a very narrow range of partial pressure of hydrogen that may influence the end product formation pattern of this strain.

Strain J4, the other *Thermoanaerobacter* strain, was on the other hand not producing high amounts of ethanol under "standard conditions", but mainly directing the carbon flow to acetate and the electrons to hydrogen. Thus, from one mole of glucose the strain is producing about 1.0 mol of acetate and more than 2.0 mol of hydrogen. By including thiosulfate in the culture the glucose-acetate ration remains the same (1:1) but increases to

1:1.6 with the methanogenic co-culture. Thus, addition of electron scavenging systems does not change the carbon flow for this strain dramatically but enhances glucose degradation and acetate formation.

The *Paenibacillus* strain J2 is a very poor hydrogen producer in general. The general stoichiometry of glucose degradation is that from one mole of glucose the strain produces 1.2 mol ethanol, 0.58 mol acetate and 0.3 mol hydrogen. The presence of thiosulfate or methanogen in the culture does not affect the end product formation of the strain. Most species within the genus *Paenibacillus* are facultative aerobes and probably contain pyruvate formate lyase instead of pyruvate ferredoxin-oxidoreductase as strict anaerobic bacteria do (Jones, 2008).

The *Clostridium* strain (J3) produces mainly acetate and butyrate as volatile end products together with hydrogen and carbon dioxide. This strain was not affected by electron scavenging systems and produced similar amounts of acetate and butyrate from all three culture conditions. This is a little surprising since the strain is a very good hydrogen producer. One explanation might be the different metabolic pathways this strain uses as compared with J4, i.e. the production of a mixture of acetate and butyrate instead of producing only acetate as volatile end product.

5.7 Production of ethanol and hydrogen from complex biomass

A large focus was towards the production of both hydrogen and ethanol from lignocellulosic biomass in present investigation. Production of biofuels from lignocellulosic biomass has gained increased interest in recent years. Various pretreatment methods and types of biomass have been used. Most of the biomass has been pretreated with dilute sulfuric acid or with alkaline pretreatment as was done in the present investigation. Table 3 and Table 5 show some selected data of ethanol and hydrogen production from various types of biomass by thermophilic bacteria. The maximum yield of ethanol from the fermentation of glucose is 2 mol ethanol per mol hexose or 11.1 mM per gram biomass. Ethanol production from Whatman paper by two of the highly ethanol producing strains show that strain J1 is producing maximum 7.13 mM/g and strain J2 is producing maximum of 4.44 mM/g dry weight. In both experiments there are lower yields observed on cellulose

pretreated with alkali, which may have been caused by some inhibitory compounds prevailing in this pretreatment method. These values on cellulose are slightly lower than observed for *Thermoanaerobacter* AK17 (Almarsdottir *et al*., 2012) and *Thermoanaerobacter* BG1L1 (Georgieva & Ahring, 2007a; Georgieva *et al*., 2008).

When cultivated on lignocellulosic biomass types, much lower yields were observed. Highest yields of ethanol for both "ethanol" strains was on grass pretreated with acid; 3.29 mM per g grass for strain J1 and 2.25 mM per g grass for strain J2. The lowest yields were observed on alkali pretreated hemp leaf; 0.55 mM per g biomass for strain J1 and 0.04 mM per g biomass for strain J2. Usually, acid and alkali enhanced ethanol yields on the lignocellulosic biomass. The only exception is on hemp, both leaves and stem. This is a little surprising since other studies with thermoanaerobic bacteria on this substrate show much higher values of ethanol when pretreated with acid or alkali (Almarsdottir *et al*., 2012; Sigurbjornsdottir & Orlygsson, 2012; Orlygsson, 2012). One explanation might be due to the fact that the hemp used in this investigation was approximately 4 years old and may have lost some of its carbohydrates and proteins. The only substrate showing high yields of liberated hexoses with the alkali pretreatment was the straw, which is most probably due to high lignin content of this type of biomass.

The two hydrogen producing strains (J3 and J4) produced maximum 6.04 mM per g Whatman paper (J3) and 5.06 mM per g Whatman paper (J4). The maximum theoretical yield of hydrogen is 4 mol hydrogen per mol glucose equivalent, as mentioned before. Thus, from 1 g of cellulose the maximum yields of hydrogen are 24.68 mmol/L if only acetate, hydrogen and carbon dioxide would be produced. Strains J3 and J4 produce much less than these values. The reason for this is because of other end products (e.g. butyrate) or incomplete substrate degradation. This experiment was performed with L/G ratio of 1:1 which also explains lower hydrogen yields as observed on Figures 37 and 38. As observed for ethanol yields for strains J1 and J2, the hydrogen producing strains (J3 and J4) also showed lower yields on lignocellulosic biomass as compared to pure cellulose. Also, inclusion of chemical pretreatment increased hydrogen yields on this type of biomass and as observed most strikingly with acid pretreated straw for strain J2 (more than twofold increase) and alkali pretreated straw (twofold increase).

5.8 Biomass pretreatment methods

As discussed thoroughly in chapter 2.5, numerous different pretreatment methods have been described which have the main purpose to disrupt the lignocellulotic structure of complex biomass and make sugar polymers for accessible for enzymatic hydrolysis. In fact, the suitability of different pretreatment methods is very hard to compare - because the whole process of converting complex biomass into biofuels has to be considered in order to find the most appropriate pretreatment method for each occasion. The performance of each pretreatment method depends on the type and composition of the biomass used. Furthermore, the microorganisms used for biofuel production have different tolerance to the various types of inhibiting compounds which originate to the biomass pretreatment, but the types and amounts of these compounds depend strongly on the pretreatment method used (Galbe & Zacchi, 2007).

In the present study, only two types of pretreatment methods were used; dilute acid pretreatment and alkali pretreatment. These pretreatment methods had different effect on the biomass types. Very weak effect was observed for newspaper, hemp leaves and hemp stem. For all the four strains, the pretreatment methods had negative effect of biofuel production from Whatman paper. In contrast, a positive effect of both the methods was observed for all the strains except for J3, where the effects were only minor. Interestingly, pretreatment with base had more positive effect on biofuel production from straw than dilute acid pretreatment. This is possibly due to the high lignin content of straw, which has been reported in literature (Almarsdottir *et al.*, 2012).

Samples of two similar types of timothy grass, "Akramýri" and "Tjarnarspilda", were analyzed thoroughly in this study. Among the experiments performed was a total sugar analysis on hydrolysates from the two grass types, pretreated in different ways. According to this experiment, dilute acid pretreatment is more suitable for timothy grass than alkali pretreatment in terms of the amounts of monosugars present in the deriving hydrolysate. For "Akramýri" timothy grass, hydrolysate with 25 g/L solid loading pretreated with dilute acid contained 43.4 mM glucose, 51.4 mM xylose and 5.5 mM arabinose. In comparison, the alkali pretreatment resulted in hydrolysate containing 40.6 mM glucose, 34.5 mM xylose and 3.3 mM

arabinose. Interestingly, when compared with the alkali method, more xylose was observed when no pretreatment method was used (38.7 mM glucose, 40.7 mM xylose, no arabinose detected). These results are in compliance with the earlier hydrolysate experiment, where slightly higher yields are observed for acid pretreatment than alkali pretreatment biomass for strains J1, J3 and J4. For strain J2, the composition of the end products changes between the pretreatment methods. However, it should be kept in mind that the suitability of different pretreatment methods involves not only the amounts of sugars present in the derivative hydrolysates. One extremely important factor is the different types and amounts of inhibitory compounds produced during the pretreatment, but this study does not take on that issue.

5.9 Fermentation of analyzed timothy grass hydrolysate

To investigate degradation of grass HL in more detail, it was decided to use two strains; one good ethanol producer (J1) and one good hydrogen producer (J4). Since these strains show a very different behavior, for example by different substrate concentrations, and the influence of the partial pressure of hydrogen these parameters were focused upon. This was done by using three different concentrations of both grass and cellulose as well as three different L/G ratios.

As expected, strain J1 was a very efficient ethanol producer and degraded most of the sugars present in both HLs, producing mainly ethanol as end product (Table 18 and Table 19). However, if ethanol yields from pure glucose degradation are calculated from 30 mM initial concentration (see Figure 22) the strain is producing about 1.7 mol from one mole of glucose. This was however considerable less in the 5 g/L Whatman paper HL (5 g/L Whatman paper corresponds to 30.9 mM of glucose assuming a total hydrolysis of the paper), or 35.5 mM which corresponds to 1.24 mol ethanol per mol glucose (corrected for glucose not utilized). The most likely reason for this difference in yields is probably due to inefficient hydrolysis of the cellulose, but unfortunately the initial glucose values given in Tables 18-21 are theoretical but not analyzed.

Regardless, the main conclusion from Whatman paper and grass HLs is the fact that J1 was not affected by increased sugar concentration and end-

product formation increased proportionally with increasing concentrations of HLs. The strain also showed stable ethanol production at different L/G ratios although acetate and hydrogen formation increased at very low partial pressure of hydrogen. The highest ethanol yields obtained in these two experiments were 1.44 mol ethanol per mol glucose equivalent on Whatman paper HL (lowest L/G used) and 0.94 mol ethanol per mol glucose equivalent on grass HL (lowest L/G used). These values are very good compared to other studies (see Table 3). The highest yields of ethanol production reported are by *Thermoanaerobacter* species. *Thermoanaerobacter mathranii* was isolated from Grændalur in Hveragerði (Iceland) and has been shown to produce 0.83 mol ethanol per mol xylose present in wheat straw hydrolysate, pretreated with wet oxidation (Klinke et al., 2001). Three *Thermoanaerobacter* species, *T. ethanolicus*, an unidentified *Thermoanaerobacter* species, and *T. thermohydrosulfuricus*, were investigated for ethanol production from beet molasses (Avci & Donmez, 2006). The concentration of sugars in the hydrolysate was 19.5 g/L and ethanol yields varied from 0.53 to 1.31 mol ethanol per mol biomass. A study on *Thermoanaerobacterium* strain AK17 where various factors were investigated with the main aim to maximize ethanol yields from lignocellulosic biomass was recently published (Almarsdottir *et al*., 2012). The authors investigated different concentration of cellulose HL and grass HL as well as different concentration of acid, alkali and enzymes during pretreatment. The main results were that the HL concentration was most important and ethanol yields at 7.5, 5.0, and 2.5 g/L of Whatman paper increased from 1.05, 1.35 and 1.55 mol ethanol per mol glucose equivalent, respectively. From grass HL this increase was from 0.86, 0.88 and 0.99 mol ethanol per mol glucose equivalent. Interestingly, strain J1 is much more tolerant to higher substrate loadings compared to *Thermoanaerobacterium* AK17, who is also inhibited at much lower glucose concentrations as compared with strain J1 (Almarsdottir *et al*., 2012).

Apart from being a good hydrogen producing strain, J4 was also chosen because of its ability to respond strongly to different partial pressure of hydrogen (see Figure 31) and its sensitivity towards relatively low substrate concentration (see Figure 24). The present study on Whatman paper and grass HL clearly show this characteristic of the strain. Strain J4 was strongly inhibited by increased hydrolysate concentration, although this

inhibition was more severe in the Whatman paper HL compared to grass HL (Table 20). This may be explained by the fact that cellulose is composed of only glucose whereas grass is composed of different types of sugars, allowing for higher percent degradation at lower HL concentrations. By using different L/G ratios (at 5 g/L concentration of HL) it was clear that the partial pressure of hydrogen strongly affected both the amount of glucose degraded and hydrogen yields (Table 21). On cellulose, the strain produced 41.3 mmol/L of hydrogen at the lowest L/G ratio but only 4.8 mmol/L at the highest ratio, but this corresponds to 1.35 and 0.19 mol hydrogen per glucose equivalent (calculated from Table 21). Highest hydrogen yields from grass HL were also observed at the lowest L/G ratio, or 38 mmol/L, but only 5.3 mmol/L at the highest L/G ratio (this corresponds to 1.23 and 0.18 mol hydrogen to mol glucose equivalent, respectively). Thus, accumulation of hydrogen in closed batch system seems to be more significant than other factors, e.g. substrate concentrations and the lowering of pH caused by increased acetate formation. Hydrogen production is generally directly in proportion with acetate formation; one mole of acetate formed should be followed by two moles of hydrogen. This is not true for most of the values observed (Table 20 and Table 21) and sometimes hydrogen is even lower than acetate. The reason for this could be caused by an active formate dehydrogenase in this bacterium and the production of formate instead of hydrogen, but this has been observed in some true anaerobes like *Clostridia* (Sparling *et al.*, 2006).

6 Conclusions

Four strains were investigated in this study. Two of the strains, strain J1 (*Thermoanaerobacter*) and strain J2 (*Paenibacillus*), were good ethanol producers with yields above 1.5 mol ethanol per mol sugar. Hydrogen was the main product of the other two strains, J3 (*Clostridium*) and J4 (*Thermoanaerobacter*). Strain J3 produced 1.0 mmol/L hydrogen per mol glucose with 20 mM initial glucose concentration and a L/G ratio of 1:1. The best hydrogen yields observed for strain J4 was on the lowest L/G ratio used (0.017), 2.5 mmol/L per mol glucose. The *Thermoanaerobacter* strains (J1 and J4) were thermophilic whereas strains J3 and J4 were observed as moderate thermophiles.

All the strains were tested on 22 different carbon substrates. None of these four strains was able to degrade any type of cellulose provided (Whatman paper, avicel, CMC). Strain J1 has the broadest substrate spectrum; degrading all mono- ,di-, and trisaccharides tested as well as serine and pyruvate. Strain J2 degraded 10 of the substrates, J3 degraded 11 and J4 degraded 14.

Heat and ethanol tolerance was tested for strains J1, J2 and J3. Strains J1 and J4 were most tolerant towards heat; both survived 100°C heat shock for more than 100 minutes. Strain J2 only tolerated 20 minutes of heat shock, but no growth was observed for 30 minutes and more. Moreover, strain J1 was least sensitive to ethanol concentrations, tolerating 4.2% ethanol. Strains J2 and J4 both tolerated approximately 3.4% ethanol.

Strain J1 observed to be very tolerant to high initial concentrations of glucose, still utilizing nearly 80% of the glucose at initial concentration of 100 mM. On the other hand, strains J2, J3 and J3 were all severely inhibited by increased substrate loadings. Investigations on the effect of partial pressure of hydrogen and electron scavenging systems (by adding either thiosulfate or hydrogenotrophic methanogen to the cultures) showed that neither of these factors changed the end product formation of strains J1 and J2 sharply. In contrast, the carbon flow of strain J4 changes dramatically by increasing substrate concentrations, changing L/G ratios or adding electron

acceptors to the media. A fundamental shift in end product formation is observed when the initial concentration of glucose is increased from 20 mM to 30 mM. Less than 5 mM ethanol is produced at low initial glucose concentrations (5-20 mM), but ethanol increases to 24.2 mM at 30 mM glucose concentration whereas the amounts of both acetate and hydrogen decrease. A similar shift is observed when the L/G ratio is increased from 0.34 to 3.27; a strong increase in ethanol production is observed whereas the amounts of both acetate and hydrogen decrease dramatically. Additionally, increased glucose concentration led to increased ethanol and less acetate by strain J4.

All the strains were able to grow on hydrolysates from different lignocellulosic biomass. The highest ethanol production observed for strain J2 was on grass pretreated with dilute acid, producing almost 15 mM ethanol. Slightly higher yields were observed for strain J1. The strain produced most ethanol on dilute acid and alkali pretreated grass as well as on alkali pretreated straw, with values between 15.7 and 16.8 mM ethanol. Strain J4 was the most powerful hydrogen producer; over 14 mmol/L of hydrogen were observed for all the biomass types used. On alkali pretreated straw, the strain produced over 20 mmol/L of hydrogen.

Samples of two similar types of timothy grass were analyzed in this study. The composition of these two types was observed to be slightly different. Hydrolysates from "Akramýri" timothy grass (25 g/L solid loading), pretreated with dilute acid, contained 43.4 mM glucose, 51.4 mM xylose and 5.5 mM arabinose. This analyzed timothy grass type was used in a second round of hydrolysate experiments, where both different hydrolysate concentrations and different L/G ratios on strains J1 and J4 were investigated. Strain J1 degraded nearly all the sugars present in the hydrolysates, regardless of initial hydrolysate concentrations and L/G ratios. The strain produced 41.8 mM ethanol at the highest hydrolysate concentration (10 g/L), but the ethanol production was not affected by the use of different L/G ratios. Strain J4 was inhibited by increased hydrolysate concentrations, which is in compliance with earlier results on initial glucose concentrations. The strain showed a strong negative correlation between L/G ratios and production of both acetate and hydrogen, as observed before.

7 References

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