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

Physiological and phylogenetic studies of *Caloramator* and *Thermoanaerobacterium* species:

Ethanol and hydrogen production from complex biomass

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"There are in fact two things, science and opinion; the former begets knowledge, the latter ignorance."

Hippocrates

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Declaration

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

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Margrét Auður Sigurbjörnsdóttir

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

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Dr. Jóhann Örlygsson

Abstract

Three anerobic, thermophilic bacteria were isolated from hot springs in Graendalur, SW-Iceland. The strains were investigated with respect to phylogenetic, physiology and end product formation.

Phylogenetic studies were done with both partial and full 16S rRNA sequence analysis on all three strains. Two of the strains, AK_{44} and AK_{35}, belong to the genus *Caloramator* with the closest relation to *C. viterbensis*. The third strain isolated, AK_{54}, falls within the genus *Thermoanaerobacterium* with the closest relation to *T. acidotolerans*. Optimum growth conditions were investigated with respect to temperature and pH. The temperature range for growth varied between the strains; 40.0 – 60.0°C for AK_{44} and AK_{35} and 55.0 – 70.0°C for AK_{54}. The pH range varied from 4.0 – 8.0 for strain AK_{44}; pH 6.0 – 8.0 for AK_{35} and 4.0 – 6.0 for AK_{54}.

The ability of utilizing various substrates was tested for all strains. All strains were saccharolytic and degraded mono- and disaccharides.

The effect of increased substrate concentration on growth and end product formation was tested. A clear inhibition was observed at increased loadings for all strains. Kinetics of glucose degradation and generation times were investigated under optimal growth conditions. End products from glucose were ethanol, acetate and hydrogen (and CO_2) for all strains.

The effect of partial pressure of hydrogen on hydrogen production was investigated for strain AK_{54}. The liquid/gas ratio clearly affected the hydrogen capacity of the strain as well as other end products produced. Hydrogen and ethanol production from hydrolysates from various complex biomasses was tested on strain AK_{54}. Different pretreatments were used.

Key words: hot spring, thermophilic, complex biomass, hydrogen, ethanol.

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First and foremost I would like to thank Dr. Jóhann Örlygsson for giving me the opportunity to work on this interesting project, giving me invaluable help and guidance. I would also like to thank Steinar Rafn Beck, Máney Sveinsdóttir and Ingólfur Bragi Gunnarsson for their help in the lab; the staff at Prokaria for helpful informations and guidance on the phylogenetics and Ásgeir Ívarsson for his helpful suggestions. Furthermore, I would like to thank my co-workers on the lab, Hildur Vésteinsdóttir and Hilma Eiðsdóttir Bakken.

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Útdráttur

Þrír loftfirrtir, hitakærir bakteríustofnar voru einangraðir úr heitum hverum í Grændal, SV-Íslandi. Stofnarnir voru rannsakaðir með tilliti til erfðafræðilegs skyldleika, lífeðlisfræði og myndunar lokaafurða.

Skyldleikarannsóknir á stofnunum voru gerðar með bæði hlut- og fullraðgreiningu á 16S rRNA. Tveir stofnanna, AK_{44} og AK_{35}, tilheyra *Caloramator* með mesta skyldleika við *C. viterbensis*. Þriðji stofninn sem einangraður var, AK_{54}, tilheyrir genamenginu *Thermoanaerobacterium* með mesta skyldleika við *T. acidotolerans*. Kjörvaxtarskilyrði m.t.t. hita- og sýrustigs voru rannsökuð. Hitastigsbilið var mismunandi milli stofna; 40.0 – 60.0°C hjá stofnum AK_{44} og AK_{35} og 55.0 – 70.0°C hjá AK_{54}. Sýrustigsbilið var frá 4.0 – 8.0 hjá stofni AK_{44}; pH 6.0 – 8.0 hjá AK_{35} og 4.0 – 6.0 hjá AK_{54}.

Hæfni stofnanna til að brjóta niður mismunandi efni var könnuð. Allir stofnarnir voru sykrusundrandi og brutu niður ein- og tvísykrur.

Áhrif aukins styrks hvarfefnis á vöxt og myndun lokaafurða var könnuð. Augljós hindrun kom fram með auknum styrk hjá öllum stofnum. Vaxtarhraði og glúkósa niðurbrot voru könnuð við kjörvaxtaraðstæður stofnanna. Lokaafurðir myndaðar úr glúkósa voru etanól, ediksýra og vetni hjá öllum stofnum, auk CO_2.

Áhrif hlutþrýstings vetnis á vetnisframleiðslu voru rannsökuð hjá stofni AK_{54}. Rúmmálshlutfall vökva/gass hafði greinileg áhrif á vetnisframleiðslu stofnsins sem og aðrar lokaafurðir sem framleiddar voru. Vetnis- og etanólframleiðsla úr hýdrólýsötum sem gerð voru úr mismunandi flóknum lífmassa var könnuð hjá stofni AK_{54}. Mismunandi formeðhöndlanir voru notaðar.

Lykilorð: heitir hverir, hitakær, flókinn lífmassi, vetni, etanól.

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1. Research objective

The objective of this research was twofold. One was to isolate anaerobic, thermophilic bacteria capable of degrading glycerol to the high-value compound 1,3-propanediol. Strains, isolated on glycerol, were investigated. Based on phylogenetic studies, two strains were selected for further studies with emphasis on their physiology and end product formation. Characterizations of both strains are presented as manuscript of scientific paper (chapter 5).

The other objective was to investigate hydrogen and ethanol production on hydrolysates made from various complex biomasses. One strain, isolated from Icelandic hot spring, was chosen for further physiological studies. Data is presented as a manuscript of scientific paper (chapter 6).

2. Introduction

Today, the world's energy demand is mostly met by fossil fuels; petroleum leading with 43% of the total consumption, followed by natural gas (15.6%) and coal (8.3%) (Environmental Literacy Council, 2008). It has been estimated that by the year 2050 the world's oil reservoir will be used up which makes the need for alternative fuels imperative (Saxena *et al.*, 2009). Fossil fuel has high sulfur, nitrogen and metal content and its burning results in extensive amount of SO_2 and NO_x being released to the atmosphere. Combustion of fossil fuels additionally releases CO_2 which is considered to have undesirable climatic consequences (Pütün *et al.*, 2001).

Energy, derived from biomass, has gained much attention the recent years and has the advantage that it is carbon neutral since it does not contribute to any net increase in atmospheric CO_2. The CO_2 arising from renewable energy is originally bound by growing plants, making no net contribution to global warming (Wheals *et al.*, 1999; Lin & Tanaka, 2006; Byung-Hwan & Hanley, 2008).

Many renewable alternatives have been explored as biofuels, e.g. bioethanol, biohydrogen, biodiesel and biogas. Production of ethanol from corn is a well known process and is already produced in the U.S. on a large scale (Angenent, 2007). However, corn derived ethanol has disadvantages, leading to increased interest in production from more complex biomass which can be used as a source for both bioethanol and biohydrogen, but is not yet economically feasible and cannot compete with the cost of gasoline (Olsson & Hahn-Hägerdal, 1996).

Hydrogen is an energy carrier, like electricity or gasoline, and can be used as fuel although it is still at development stage (Schlapbach & Züttel, 2001). Presently, hydrogen is most commonly produced from fossil fuels but other feedstocks are possible, e.g. biomass (Das & Veziroğlu, 2001).

Microbial fermenations are potential alternative to produce bioethanol and biohydrogen. Various kinds of biomass can be used as feedstock for the production, there among lignocellulosic biomass (Zaldivar *et al.*, 2001).

2.1 Ethanol

Ethanol is a colorless, flammable and volatile liquid with boiling point at 78.4°C and melting point at -112.3°C (Kosaric & Vardar-Sukan, 2001). Ethanol has a hydroxyl group and can therefore participate in hydrogen bonding. This makes ethanol more viscous and less volatile than other organic compounds of similar molecular weight (Moore *et al.*, 2005).

The production of ethanol has a long history and has been dated as far as 7000 B.C. in China (McGovern *et al.*, 2004). In addition to alcoholic beverages, ethanol is used in cleaning agents, cosmetics, as a antifreeze agent and fuel supplement, in chemical industry and as feedstock in the synthesis of several chemicals like acetic acid and acetaldehyde (Kosaric & Vardar-Sukan, 2001).

Ethanol can be used as vehicle fuel but compared to gasoline, it has lower energy per unit volume; 23.60 and 34.85 gJ m^3, respectively (Das, 1996). Despite this, ethanol has low toxicity and better fire safety, and has thus been considered to be safer than gasoline (Kosaric & Vardar-Sukan, 2001). Cars, fueled by ethanol, were first developed in the 1880's by Henry Ford when he designed the early Ford Model T that used ethanol made from corn (Zaldivar *et al.*, 2001). Ethanol was used as fuel for vehicles into the 1930's, however, fossil fuels quickly dominated the market early in the 20th century and low gasoline prices persisted for several decades. The oil crisis in the 1970's led to renewed interest in ethanol and the importance of alternative energy sources was underlined (Zaldivar *et al.*, 2001). In Brazil, this led to the program PRO-ALCOOL, which resulted in the developement of efficient alchol burning engines. In the 1980's, more than 90% of vechicles sold in Brazil were running on ethanol (Kosaric & Vardar-Sukan, 2001).

2.1.1 Bioethanol

Bioethanol has gained much attention in recent years as biofuel. Compared to gasoline, it has higher octane number, broader flammability limits, higher flame speeds and higher heat of vaporization and is thus theoretically more efficient than gasoline (MacLean & Lave, 2003). Disadvantages, apart from lower energy density,

are that ethanol can be corrosive, has low flame luminosity and lower vapor pressure which makes cold starts difficult (Balat *et al.*, 2008).

Vehicles can run on 100% ethanol but usually it is blended with gasoline. The most common blends are E10, E20, E30, E40 and E85. The E10 blend consists of 10% ethanol and 90% unleaded gasoline. About 70% of gasoline in America contains ethanol, mostly as E10 and vehicles can use the blend without modifying the engine but the higher concentrations, ethanol cannot be used in unmodified vechicles (American Coalition for Ethanol, 2009). Car manufacturers have developed flexible fuel vehicles (FFV) that can run on the E85 blend and now over six million FFVs have been sold in the U.S. (DOE, 2006). If E85 is not available, FFV can operate on any ethanol blend up to 85% or even pure gasoline (American Coalition for Ethanol, 2009).

2.1.2 Production of bioethanol

Many countries are producing bioethanol and the production has increased remarkably in recent years. The world's annual production has reached about 51.000 million liters (Renwable Fuels Association, 2009) with the U.S. (18.000 million liters) and Brazil (17.000 million liters) as the largest producers, followed by China and India with much less production (4.000 million and 2.000 million liters, respectively). In Europe the largest producers are France and Germany with 950 million and 800 million liters, respectively (Sánchez & Cardona, 2008).

Ethanol can be produced from a great variety of sources, i.e. corn, sugar cane and lignocellulosic biomass (Wheals *et al.*, 1999) but about 90% of the world's production is based on sugar or starch based crops by fermentation (referred to as first generation ethanol), the rest is produced chemically. Fuel ethanol produced in the U.S. is from corn but sugar cane is the main raw material for production in Brazil, India and South Africa (Zaldivar *et al.*, 2001). This type of raw material has been strongly debated since it is competing with the feed and food application, causing less food supply and thus higher food prices. Interest in second generation bioethanol production is therefore rising, where fermentation of more complex biomass is used e.g. lignocellulosic biomass (Hahn-Hägerdal *et al.*, 2006). This kind of biomass consists of sugars, both pentoses and hexoses, with more complex structures and is

therefore not easily degradeble. Lignocellulosic biomass is not used for food and includes agriculture waste (wheat straw, corn stalks, soy bean residues, sugar cane bagasse), industrial watste (from pulp and paper industry), forestry residues and municipal solid waste (Lin & Tanaka, 2006; Zaldivar *et al.*, 2001).

2.1.2.1 Ethanol producing microorganisms

Today, ethanol is most commonly produced by *Saccharomyces* yeasts, particularly *S. cerevisae* (Gray *et al.*, 2006). The yeast degrades glucose through glycolysis to high yields of ethanol and CO_2. Two moles of ATP are produced during the degradation (Glazer & Nikado, 2007). The theoretical ethanol production from one mol of glucose are two moles (Olsson & Hahn-Hagerdal, 1996) and *S. cerevisae* produces about 95% of theoretical yield (Glazer & Nikado, 2007). However, this microorganism is not well suited for fermentation of lignocellulosic biomass since one of the main sugar released when hemicellulose is hydrolysed is xylose which is undegradable by wild type yeasts (Dien *et al.*, 2003).

Another well known ethanol producing microorganism is the Gram-negative bacterium *Zymomonas mobilis*. It is an osmo- and ethanol-tolerant bacterium with high growth rate and specific ethanol production under anaerobic conditions (Cazetta *et al.*, 2007). However, this bacterium can only ferment glucose, fructose and sucrose. The metabolism of each sugar has distinctive features through different pathways (Entner Doudoroff pathway), and it is likely that it has not been well suited for fermentation of complex biomass (Gray *et al.*, 2006; Glazer & Nikado, 2007). Genetic engineering has been done on both *Z. mobilis* and *S. cerevisae* in order to broaden their substrate spectrum utilization. Two operons (xylose isomerase and xylulokinase) and pentose phosphate pathway enzymes were transformed into *Z. mobilis*, resulting in fermentation of xylose as carbon source and ethanol production of 0.44 g g^{-1} xylose (86.2% of theoretical yield). Research has been done on flocculated *S. cerevisae* strain for ethanol production from acid hydrolysate from wood biomass. Result showed that the strain was able to consume the glucose at high rates. Mannose and galctose were also consumed but the concentration of xylose was the same at the beginning and end

of the incubation time which suggests no consumption despite genetic modification on the strain (Tang *et al.*, 2006).

For microorganism to be well suited for ethanol production on industrial scale it should have the following properties: (Dien *et al.*, 2003; Zaldivar *et al.*, 2001)

- High ethanol yield per unit substrate (> 90% of theoretical yield)
- Broad substrate range
- High ethanol tolerance (> 4%)
- Minimal by-product formation
- High ethanol productivity (> 1 g l^{-1} h^{-1})
- Simple nutrient requirments

Many bacteria, both mesophilic and thermophilic, are known ethanol producers. Among those are *Thermoanaerobacter ethanolicus*, *Clostridium saccharolyticum*, *Clostridium thermocellum*, *Escherichia coli* (mutant strain), *Thermoanaerobacterium aotearoense* and *Thermoanaerobacter mathranii* (Classen *et al.*, 1999; Lin & Tanaka, 2006; Liu *et al.*, 1996; Larsen *et al.*, 1997). *T. ethanolicus* can be regarded as strict ethanol producer with 1.9 mol EtOH per mol glucose utilized, the highest yield reported for thermophilic bacteria (Wiegel & Ljungdahl, 1981). Bacteria belonging to the genus *Caloramator* are also able to produce ethanol, however, they have not been reported for high ethanol yields (Seyfried *et al*., 2002).

2.2 Thermophiles

The four main environmental factors that affect the activity of microorganisms are temperature, pH, water availability and oxygen. Temperature is one of the most important factors affecting microorganisms' growth. According to common thermodynamics, chemical and enzymatic reactions proceed at more rapid rates at higher temperature, causing faster growth. However, if the temperature is too high, proteins will denaturize and can be damaged. Each species therefore has a minimum, a maximum and an optimal temperature for growth. The most rapid growth is at the optimum temperature which is generally closer to the maximum than the minimum.

In relation to the optimum temperature it is possible to divide bacteria into four groups: *psycrophiles* (optima 4°C), *mesophiles* (39°C), *thermophiles* (60°C) and *hyperthermophiles* (88°C). Mesophiles can be found in warm-blooded animals but psycrophiles and thermophiles are in cold or hot environments. Hyperthermophiles are found in extremely hot habitats, such as hot springs and geysers (Madigan *et al.*, 2003). Additionally, there is a group of archaea which have higher optimum temperature than the bacteria. The archaeas can survive at extreme conditions in relations to temperature, high salt concentration or even under great atmospheric pressure. They form complex ecosystems that consist of variety of primary producers and decomposers of organic matter. One of these organisms is the archaea *Pyrolobus fumarii*, the most extreme thermophile isolated yet, which has a temperature optimum at 106°C. This archaea is so well adapted to high temperatures that it does not grow at 85°C or below (Huber & Stetter, 1998).

There is a great variety in the habitat of thermophiles but there are mainly four factors that generate thermal environment, i.e. solar heating, combustion processes, geothermal activity and radioactive decay. Solar heating can lead to soil temperatures at 60°C although such conditions occur only during the daylight hours. High temperature environment is probably mostly caused by geothermal activity. Although the temperature in active volcanoes are too high for living organisms, hot springs and fumaroles associated with volcanic activity have more reasonable temperatures for development of thermophilic organisms (Brock, 1978).

The ability of thermophiles to live at high temperatures is mainly due to their thermostable proteins. Most proteins from mesophiles denature at temperatures below 100°C (Perry *et al.*, 2002). The thermostability of the enzymes includes hydrogen bonding, hydrophobic bonding, ionic interactions, and metal binding and disulfide bridges. It is possible to enhance thermostability only by achieving minor changes in the primary structure, e.g. a single amino acid substitution. This has been performed in *E. coli*, whereas glutamate at position 49 was replaced by methionine resulting in increased thermostability of tryptophan synthetase in *E. coli* by increasing hydrophobic bonding (Thomas & Kenealy, 1986).

The cell membrane of thermophilic bacteria contains more saturated fatty acids which make it stiffer and more thermostable as compared to mesophiles. The cell

membrane of hyperthermophiles is made of repeated five carbon isoprene units instead of fatty acids and is linked by ether linkage to glycerol phosphate. This structure makes the membrane more heat resistant than the lipid bilayer of species of bacteria and eukaryotes (Madigan *et al.*, 2003). Enzymes from thermophiles are attractive for biotechnological applications because of their thermostability. One of those enzymes is DNA polymerase which is used for polymerase chain reaction (PCR) – one of the most used enzymes in genetic engineering today. The enzyme was isolated from the thermophile *Thermus aquaticus*, isolated from a hot spring in Yellowstone, USA (Huber & Stetter, 1998).

2.2.1 Anaerobic microorganisms

All organisms need energy for life. The energy can be gained in three ways; through organic chemicals, inorganic chemicals or light. The energy is obtained by oxidation and is conserved in the cell as the high energy compound *adenosine triphosphate* (ATP). Microorganisms capable of extracting the energy only in presence of oxygen are called *aerobes* and those that can extract the energy only in absence of oxygen are *anaerobes* (Madigan *et al.*, 2003). Anaerobic bacteria are divided into two groups; those that are inhibited or killed by oxygen are *obligate* anaerobes and those that are capable of gaining energy by aerobic respiration if oxygen is present but are also capable of switching to fermentation are *facultative* anaerobes. (Perry *et al.*, 2002).

The high-energy compound ATP is composed of ribose bound to adenine and a chain of three phosphate groups. This compound is the energy currency of the cell and can be gained in presence or absence of oxygen, depending on the organism (Johnson, 2001).

2.2.2 Geothermal areas

Geothermal areas are rare around the world and are limited to places where volcanic activity is present. Geothermal areas can be divided as following:

- Hot springs with temperature near boiling point.
- Hydrothermal vents in the ocean with temperature near 350°C or even higher.

- Steam vents with temperature up to 500°C (Madigan *et al.*, 2003).

Thermophilic bacteria are able adapt to conditions in the environment and are thus able to thrive in geothermal areas although the temperature may be slightly higher than their optimum temperature for growth. Geothermal areas offer stability in heat and are thus favorable habitats for thermophilic bacteria (Kristjánsson & Alfreðsson, 1986). In addition, communities of bacteria and archaea have been found in subterranean hot springs and boreholes (Kimura *et al.*, 2005). In general, most of the known species of thermophiles are obligate or facultative anaerobes because geothermal areas have very low oxygen concentrations. However, aerobic and microaerobic isolates are also known (Amend & Shock, 2001).

Terrestrial hot springs are found all around the world, but the main areas are in the western U.S., New Zealand, Iceland, Japan, Italy, Indonesia, Central America, Russia and Central Africa (Madigan *et al.*, 2003).

Iceland is considered to be one of the largest geothermal area in the world with difference in physical and chemical composition of the hot springs. The geothermal areas in Iceland can be categorized as following:

- **High temperature areas** are located within active volcanic areas. There are approximately 30 known places and cover few hundreds of km^2 (Arnórsson, 1995). These places are in general sulfuric and clayish hot springs and steam vents (100 – 200°C at depth of 1000 m). Hot springs at high temperature areas are usually acidic (pH 2 – 4), and thus the concentration of dissolved chemicals are high (Kristjánsson & Alfreðsson, 1986).
- **Low temperature areas** are located on the border of active volcanic areas. The hot springs are clear water springs and pools with temperature varying from 20 – 100°C. These hot springs have high pH (8 – 10) and silica can be seen around them (Kristjánsson & Alfreðsson, 1986).

2.2.3 Microbial flora of Icelandic geothermal areas

The microbial flora of Icelandic geothermal areas has been investigated to some extent and many species have been isolated, both anaerobic and aerobic (Koskinen *et al.*, 2008a; Örlygsson & Baldursson, 2007; Sommer *et al.*, 2004).

Many species of anaerobic archaea and bacteria have been isolated from high temperature areas, such as *Thermoproteus, Thermophilum*, *Desulfurococcus* and *Sulfolobus* (Kristjánsson & Alfreðsson, 1986). Studies have shown that the variation of species is lower at high temperature areas, where the environment is extreme, compared to low temperature areas where the hot springs are neutral to alkaline (Marteinsson *et al.*, 2004). Organisms found at low temperatures areas, where the hot springs are alkaline with temperature from 30 – 45°C are mainly photoautotrophic cyanobacteria and algae (Sonne-Hansen & Ahring, 1997). As the temperature increases the microbial diversity and only two genera of cyanobacteria are present at above 45°C, *Mastigocladus* and *Phormidium*. Aerobic phototrophs like *Cloroflexus* and *Thermus* are common in hot springs where the temperature is between 50 and 85°C, but between 60 to 80°C bacteria belonging to *Aquificales* are often dominating (Kristjánsson & Alfreðsson, 1986).

Figure 1. Example of hot springs in Iceland from where anaerobic bacteria have been isolated.

2.2.4 Fermentation

Fermentation and respiration are the two main energy metabolisms of organisms that use chemicals as electron donors. In both cases ATP is synthesized but the main difference is that in fermentation no oxygen is used (Madigan *et al.*, 2003).

The first stage of extracting energy from glucose is the same in fermentation and respiration. This stage is called *glycolysis* and is a 10-reaction pathway that produces ATP by substrate-level phosphorylation (SLP). Glycolysis takes place in the cytoplasm of the cell and is not bound to any membrane or organelle. In the first step, two ATP molecules are used. Later on, four ATP molecules are formed by SLP which leads to a net yield of two ATP molecules for each glucose molecule degraded. Four electrons are also harvested as NADH and can be used to form ATP through respiration (in the presence of oxygen). When glycolysis is completed, two molecules of pyruvate are formed (Johnson, 2001). *Oxidative phosphorylation* (OP) is the process in respiration where the ATP is formed by difference in concentration of protons inside and outside the cell membrane (*proton motive force*). Fermentation releases relatively small amount of energy compared to respiration because the end products are only produced to regenerate NAD^+ (Perry *et al.*, 2002).

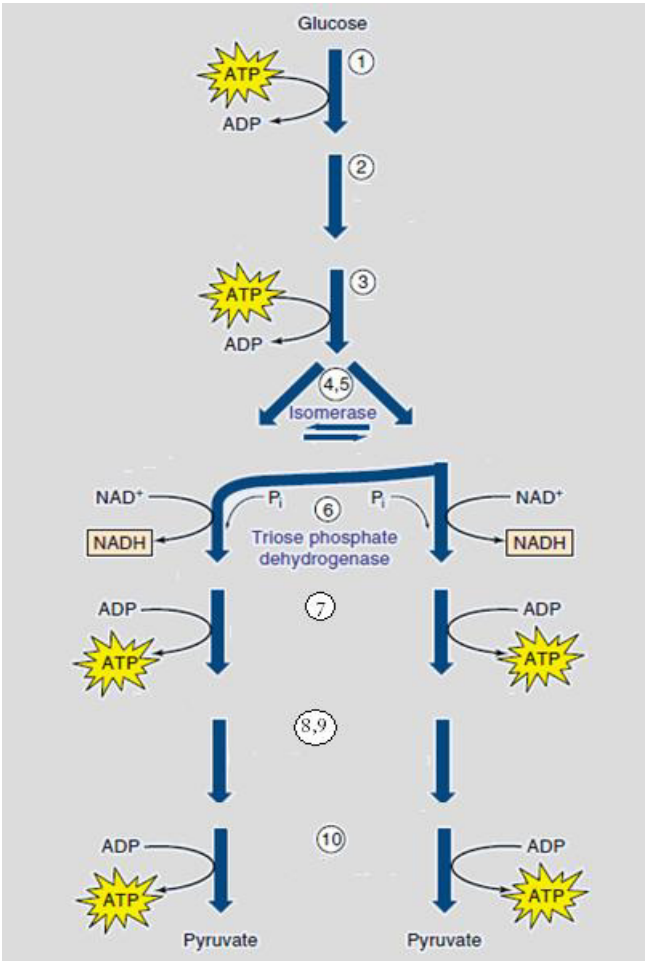

Figure 2. The glycolysis pathway (modified from Johnson, 2001).

2.3 Hydrogen production

Hydrogen is a colorless, odorless, flammable and nontoxic gas at atmospheric temperature and pressure (Universal Industrial Gases, Inc, 2003). Hydrogen has very low specific gravity (Moore *et al.*, 2005) and is approximately one-fifteenth as heavy as air (Universal Industrial Gases, Inc, 2003).

Hydrogen has a long history; it was identified as a chemical element in 1766 by Henry Cavendish (Moore *et al.*, 2005). Research of hydrogen was already active in the 1920's and 1930's when Rudolf Erren (German engineer) converted the internal combustion engines of trucks, buses and submarines to use hydrogen or hydrogen mixtures as a fuel. Due to low prices of oil and gasoline after the World War II the research in the hydrogen field halted until the energy crisis in 1970's. In 1990 the first solar powered hydrogen production plant became operational in Germany – followed by rising interest in hydrogen and growing public awareness of environmental impacts of fossil fuel (National Hydrogen Association, 2008).

When hydrogen is combusted the main product is water. Thus, hydrogen is regarded as a clean non-polluting fuel and relatively harmless to humans and the environment compared to other gaseous and volatile fuels (Das & Veziroğlu, 2001). Hydrogen has high energy yield (122 kJ g^{-1}) (Ming *et al.*, 2008) which is 2.75 times higher than hydrocarbon fuels. However, the volumetric energy density of hydrogen is low, because hydrogen contains small amount of energy by volume compared to other fuels. For that reason, final delivery of hydrogen is costly and storage in vehicles not practical; i.e. 4 kg of hydrogen takes up to 45m^3. Many ideas are now under investigation in order to overcome this problem and hydrogen storages, based on metal hydrides, have been developed (Schlapbach & Züttel, 2001).

Hydrogen can be produced from fossil fuels, biomass and water. Presently, hydrogen is most commonly (> 90%) produced from natural gas or light oils by steam reforming (Das & Veziroğlu, 2001; Levin *et al.*, 2004). However, hydrogen production by bacterial fermentation has been known since in the late 1920's (Strickland, 1929) and in the 1940's with microalgae (Gaffron & Rubin, 1942). Biological processes of hydrogen production are divided in four groups: direct

biophotolysis, indirect biophotolysis, photo-fermentation and fermentation (Manish & Banerjee, 2007).

2.3.1 Direct biophotolysis

In direct biophotolysis, hydrogen is produced from water by using solar energy to convert water to oxygen and hydrogen by following general reaction (Levin *et al.*, 2004; Hallenbeck & Benemann, 2002):

$$\mathbf{2H_2O \rightarrow 2H_2 + O_2}$$

There are two photosynthetic systems responsible for this process: photosystem I (PSI) that produces reductant for CO_2 reduction and photosystem II (PSII) that splits water and evolves oxygen (Ni *et al.*, 2006). Two photons are used for electron removal from water and used in CO_2 reduction or formation of hydrogen if hydrogenase, the enzyme that catalyzes hydrogen production, is present (Das & Veziroğlu, 2001).

Green algae and *Cyanobacteria* (blue-green algae) are among organisms that contain hydrogenase and thus are able to produce hydrogen. Electrons are generated when PSII absorbs light energy and are then transferred to ferredoxin by PSI. Hydrogenase serves as an electron acceptor and hydrogen is produced (Ni *et al.*, 2006). An example of this is the green alga *Chlamydomonas reinhardtii* which was able to produce 7.95 mmol H_2^{-1} L^{-1} after 100 hour cultivation (Levin *et al.*, 2004). However, this process is limited and only possible under special conditions because of the oxygen sensitivity of the hydrogenase enzyme (Manish & Banerjee, 2007).

2.3.2 Indirect biophotolysis

Cyanobacteria are also able to produce hydrogen through indirect biophotolysis by following reactions (Levin *et al.*, 2004; Ni *et al.*, 2006):

$$\mathbf{12H_2O + 6CO_2 \rightarrow C_6H_{12}O_6 + 6O_2}$$

$$\mathbf{C_6H_{12}O_6 + 12H_2O \rightarrow 12H_2 + 6CO_2}$$

Thus, indirect biophotolysis involves two separate steps where hydrogen and oxygen are produced, coupled through CO_2 fixation (Manish & Banerjee, 2007). This circumvents the sensitivity of the hydrogenase enzyme which is an advantage (Hallenbeck & Benemann *et al.*, 2002). An example of organism capable of hydrogen production through indirect biophotolysis is the heretocystous cyanobacteria *Anabaena variabilis* which produces hydrogen in spatially separated heterocysts by nitrogenase activity (Kotay & Das, 2008; Benemann, 1996).

2.3.3 Photo-fermentation

Photo-fermentation is well known among purple non-sulfur bacteria; they produce hydrogen by using light energy and organic acids by the following reaction (Levin *et al.*, 2004):

$$\mathbf{C_6H_{12}O_6 + 12H_2O \rightarrow 12H_2 + 6CO_2}$$

Organic acids are produced during anaerobic digestion of organic wastes and can be converted to hydrogen and CO_2 by photosynthetic bacteria (Kapdan & Kargi, 2006). Among bacteria that have been investigated for their hydrogen production are *Rhodobacter spheroides*, *Rhodobacter capsulatus* and *Rholdovulum sulfidophilum* (Koku *et al.*, 2002; He *et al.*, 2005; Matsunaga *et al.*, 2000).

The hydrogen production is catalyzed by the enzyme nitrogenase. Under anaerobic conditions these bacteria use organic acids, for example acetic acid, as electron donors. Electrons are transferred to the nitrogenase and energy in form of ATP is used. The nitrogenase reduces proton into hydrogen, again using energy in form of ATP (Manish & Banerjee, 2007).

2.3.4 Fermentation

Hydrogen production with anaerobic bacteria is a relatively simple process and wide spectrums of substrates are potential, e.g. carbohydrates, lipids, proteins and waste products, although carbohydrates are preferred. Carbohydrates predominantly give rise to acetic and butyric acids as well as hydrogen gas (Claassen *et al.*, 1999). The substrate is degraded by oxidation and metabolic energy for growth gained. During the

oxidation, electrons are generated which need to be disposed. In anaerobic environment, where no oxygen is present to serve as an electron acceptor, molecular hydrogen and reduced end products are produced (de Vrije & Claassen, 2003).

When glucose is fermented to hydrogen, pyruvate, generated by glycolysis, is oxidized to acetyl CoA. ATP is derived from acetyl CoA and acetate is excreted. This requires ferredoxin (Fd) reduction which is oxidized by a hydrogenase, leading to released electrons in the form of molecular hydrogen (Hallenbeck & Benemann, 2002).

Fermentation of carbohydrates, such as glucose, yields the highest hydrogen production. If glucose was completely converted to hydrogen the theoretical hydrogen yield would be 12 mol of hydrogen per mol glucose. However, this is never obtained in known biological systems since hydrogen is only produced in combination with volatile fatty acids or alcohols (Westermann *et al.*, 2007). The maximum production of hydrogen is 4 mol per mol glucose degraded when acetic acid is the sole end product (Ni *et al.*, 2006):

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

If butyric acid is the only end product the theoretical value decreases to 2 mol of hydrogen per mol glucose utilized (Hawkes *et al.*, 2002):

$$\mathbf{C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2H_2 + 2CO_2}$$

Degradation of carbohydrates to lactate and ethanol does not favour hydrogen production (Kim *et al.*, 2006b; Thauer *et al.*, 1977). Thus, the highest yield of hydrogen is obtained when acetic acid is the only end product formed. However, this yield is almost never achieved because the end products are usually a mixture of acetic acid, butyric acid or other end products like ethanol or lactic acid (Classen *et al.*, 1999).

About 1 – 2 moles of hydrogen per mol glucose is frequently obtained from mesophilic and moderate thermophilic fermentation (Rachman *et al.*, 1998) while hyperthermophiles produce more than 3 moles of hydrogen per mol glucose. This has

been obtained for *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii* (van Niel *et al.*, 2002). High yields of hydrogen have also been achieved from thermophilic enrichment culture from Icelandic hot springs; the production was 3.2 mol hydrogen per mol glucose in semi continuous batch reactor and 2.10 mol H_2 per mol glucose in batch culture (Koskinen *et al.*, 2008a; Koskinen *et al.*, 2008b).

2.3.5 Hydrogenases

As mentioned before, electron balance is kept stable with hydrogen production in most fermentation pathways whereas protons, originating from water, play the role of electron donors since no oxygen is present (Madigan *et al.*, 2003). Hydrogenases catalyze the chemical reaction (Manish & Banerjee, 2007; Vignais & Colbeau, 2004; van Haaster *et al.*, 2005):

$$\mathbf{2H^+ + 2e^- \leftrightarrow H_2}$$

Three enzymes carry out this reaction;

- **Hydrogenase** that is both reversible and classical. These oxidize ferredoxin or other low redox electron carriers in reactants.

- **Uptake hydrogenase** is able to take up hydrogen when partial pressure is low. High-potential electron acceptors are reduced but little or no hydrogen is produced.

- **Nitrogenase** reduces nitrogen (N_2) to ammonia but is also capable of producing hydrogen if N_2 is not present. However, it is inefficient to produce hydrogen because it costs 4 ATP to produce one mol of H_2. (Das & Veziroğlu, 2001)

These enzymes are all metalloenzymes, i.e. they have metallo-cluster structure on their active sites. According to the metal composition, hydrogenases are divided into three

distinct classes: NiFe hydrogenases (the most abundant), FeFe hydrogenases and metal – free hydrogenases. (Hallenbeck & Benemann, 2002; Kovács *et al.*, 2006; Vignais & Colbeau, 2004).

2.3.6 Hydrogen producing bacteria

A great variety of microorganisms are capable of hydrogen production. The most studied bacteria are *Clostridia* and enteric bacteria (de Vrije & Claassen, 2003). Two different enzymatic pathways are used for the metabolism of pyruvate by clostridia and enteric bacteria. Enteric bacteria use pyruvate formate lyase but clostridia use pyruvate ferredoxin oxidoreductase systems. In general, hydrogen production among these bacteria as well as others, is driven by breakdown of pyruvate which results in formation of acetyl-CoA which is then used to form ATP and either formic acid (enteric bacteria) or reduced ferredoxin (clostridia and strict anaerobes), from which hydrogen is produced (Hallenbeck & Benemann, 2002). Incomplete degradation of formate and formation of lactate by enteric bacteria results in lower hydrogen yields as compared to clostridia (Hallenbeck, 2005).

Among studied hydrogen producing bacteria are both obligate and facultative anaerobes including e.g. *Caldicellulosiruptor saccharolyticus*, *Clostridium butyricum*, *Pyrococcus furiosus*, *Ruminococcus albus*, *Thermoanerobacterium thermosaccharolyticum*, *Thermotoga elfii*, *Citrobacter intermedius* and *Escherichia coli* (van Niel *et al.*, 2002; Saint-Amans *et al.*, 2001; Schäfer & Schönheit, 1992; Ntaikou *et al.*, 2008; O-Thong *et al.,* 2008, Brosseau & Zajic, 1982, Turcot *et al.,* 2008).

2.4 Biomass

2.4.1 Starch

Starch is a polysaccharide and stores energy in plants (Johnson, 2001). Starch consists of amylase, which is linear, and amylopectine, which is larger and highly branched. The glucose units in amylase are linked together with α-1,4 glycoside linkages as well as the linear chains in amylopectine but the branching points in amylopectine have α-1,6-glycoside linkages (Gray *et al.*, 2006; Stevnebø *et al.*, 2006). Each type of starch has its own shape and size of granules where the starch is packed into, e.g. the granules in potato tubers are both smooth and irregular, wherease maize granules are polyhedral and much smaller (Oksman-Caldentey & Barz, 2002).

Starch is the main feedstock for ethanol production in the U.S. and Brazil and a broad variety of starches are possible for the production, including corn, wheat, rice (most common feedstocks in the U.S.), sugarcane, molasses and cassava (most used in tropical countries like Brazil). The substrate must by hydrolyzed before fermentation in order to break down the structure and convert the starch to sugars. This is most commonly done by enzymes (endo- and exo-enzymes) or acid hydrolysis (Kosaric & Vardar-Sukan, 2001).

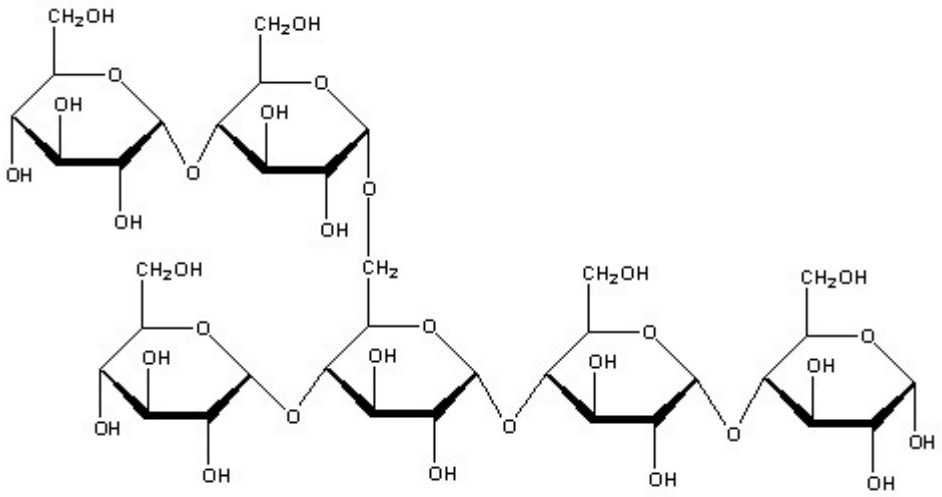

Figure 3. The main structure of starch; polymer of glucose with α-1,4 and α-1,6 linkages.

2.4.2 Lignocellulosic biomass

Lignocellulosic biomass makes up about 50% of all biomass on Earth. It consists of cellulose, hemicelluloses and lignin.

2.4.2.1 Cellulose

Cellulose is the major constituent of plants and is considered to be the most abundant organic compound present on Earth. Cellulose consists of thousands of molecules of anhydroglucose linked together β-1,4 glycoside linkages (Perry *et al.*, 2002). This is a linear homopolymer, similar to amylase except that in order to cleave the bonds in cellulose, enzymes are necessary that most higher organisms lack (Johnson, 2001).

The basic unit of cellulose is the disaccharide *cellobiose* which is rotated through 180° along the axis of the chain and the polymer appears to be ribbon like, stabilized by internal hydrogen bonds (Zaldivar *et al.*, 2001). Each cellulose unit is tightly bound to others with hydrogen bonds which cause the chain to interact strongly with one another. This makes very long crystalline aggregate, *microfibrils* which are combined to form larger fibrils. Cellulose can appear to be either crystalline or amorphous and is more resistant to degradation than other glucose polymers (Glazer & Nikado, 2007). Cellulose is generally the largest fraction in biomass, about 40 – 50% by weight (Saxena *et al.*, 2009).

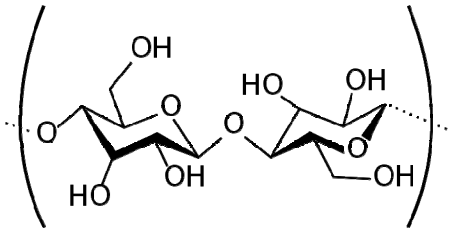

Figure 4. The main structure of cellulose (Johnson, 2001).

2.4.2.2 Hemicellulose

Hemicellulose is highly branched heteropolysaccharide, located between the microfibrils of cellulose and consists e.g. of the pentose xylose, linked by β-1,4 linkages (Evans & Furlong, 2003). Other sugars are present in the side chains of

hemicelluloses; arabinose, galactose, glucose, mannose, rahmnose, fucose as well as uronic acids and acetyl group (Glazer & Nikado, 2007). Hemicellulose forms matrixes in plants which hold the cellulose fibrils together, making the plant tissue strong and rigid (Evans & Furlong, 2003). Hemicellulose has rather weak bonds, thus, more easily hydrolyzed than cellulose (Zaldivar *et al.*, 2001).

Hemicellulose can be divided into three main groups depending on the most common polymer present: xylan (polysaccharide of xylose), mannan and galactan (Brigham *et al.*, 1996).

Softwoods (e.g. spruce and pine) and hardwoods (e.g. aspen and oak) have different structure and composition of hemicellulose (Palmqvist & Hahn-Hägerdal, 2000b). In hardwood, xylan is the dominant polymer and often represents 15 – 30% of the total dry matter content. In softwood, xylan is usually 5 – 10% (Filonova *et al.*, 2007).

Xylan polymers are present in all land plants and are therefore second in abundance to cellulose among sugar based polymers occurring in nature. Xylans can be degraded by both bacteria and fungi with two main enzymes, β-1,4-xylanases and β-xylosidases. β-1,4-xylanases hydrolyze the internal bonds that link xylose molecules and β-xylosidases release xylosyl residues. Both enzymes produce free xylose which is phosphorylated to xylose-5-phosphate and enters the pentose cycle for further degradation (Perry *et al.*, 2002).

2.4.2.3 Lignin

Lignin is the most abundant aromatic polymer on Earth, built up of phenylpropane units: *p*-coumaryl, coniferyl and sinapyl alcohol (Buranov & Mazza, 2008) that are bound together by different linkages (Hendriks & Zeeman, 2009).

Lignin can be found in cell walls of higher plants as well as ferns and club mosses but not in lichens and algae. Approximately 20 to 30% of wood and plant tissues are composed of lignin and its biodegradation is therefore an essential part of the carbon cycle. Lignin is non-water soluble and relatively indestructible and is probably not completely degraded by any single microbial species. It surrounds and protects the cellulose and hemicellulose from microbial degradation (Hendriks & Zeeman, 2009). However, lignin is degraded in nature by white-rot fungi although it is

not known to serve as growth substrate for this or any other fungi so other carbon or energy source must be available (Glazer & Nikado, 2007). Figure 5 shows the composition of plant cell wall, the cellulose hemicelluloses surrounded by lignin (modified from Thostrup, 2006).

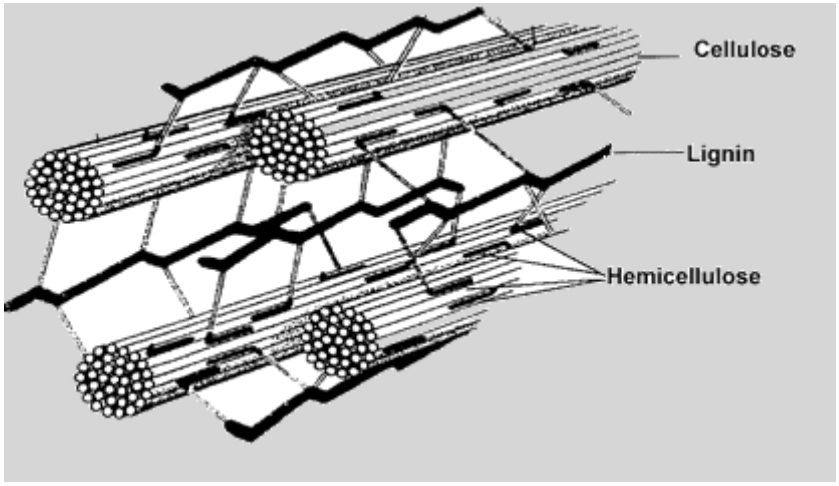

Figure 5. Composition of plant cell wall.

2.4.3 Ethanol and hydrogen production from lignocellulosic biomass

There are three main steps in the production of ethanol and hydrogen from lignocellulosic biomass. First, the polymeric biomass is broken down to monosaccharides. This step is done through physical, chemical or enzymatic methods. Next microbial fermentation of the sugars to alcohol occurs and finally the ethanol is recovered by distillation. To obtain anhydrous ethanol further distillation procedures are needed (Glazer & Nikado, 2007).

2.4.4 Pretreatments of lignocellulosic biomass

In order to make bioethanol and biohydrogen competitive to fossil fuels, low-priced feedstocks are needed to lower the production cost. Price of feedstock can contribute up to 55% of the production cost, making inexpensive lignocellulosic biomass a feasible choice. However, it is necessary to pretreat the biomass to soften the material, break down the polymeric structure and make it more susceptible to enzymes before fermentation (del Campo, Alegría *et al.*, 2006). There are many ways of pretreating biomass and the methods can be catagorized into biological, chemical, physical,

physio-chemical and others. When lignocellulosic biomass is pretreated the main goal is to degrade the lignin, increase porosity and decrease fiber cristallinity of cellulose and hemicellulose (Moiser *et al.*, 2005). Figure 6 shows schematic effect of pretreatment on biomass (modified from Moiser *et al.*, 2005).

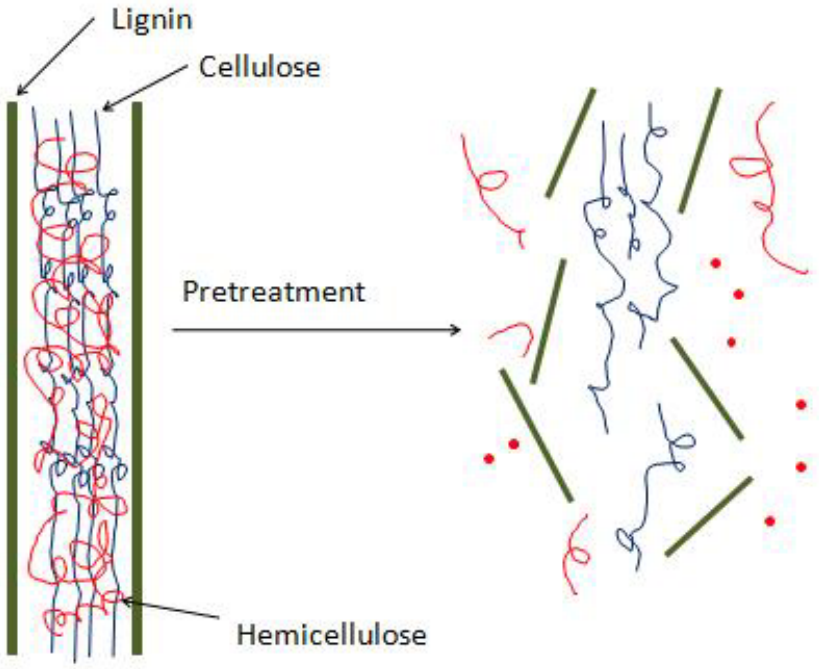

Figure 6. Schematic effect of pretreatment on biomass.

2.4.4.1 Biological pretreatment

Biological pretreatments generally include either the use of fungi to degrade the lignin or enzymes. White rot fungi have showed promising results although brown- and soft-rot fungi have also been used. White-rot fungi secrete *lignin peroxidase*, an extracellular enzyme that mediates lignin depolymerization (Glazer & Nikado, 2007). *Phanerochaete chrysosporium* is the fungi most commonly used for delignification (Rodríguez Couto *et al.*, 2003). However, the rate of lignin and hemicellulose breakdown during biological pretreatment is, in most cases, very low and needs to be optimized in order to be an effective pretreatment method (Sun & Cheng, 2002). Enzymes used for pretreatment are discussed in chapter 2.4.5.

2.4.4.2 Chemical pretreatment

Most chemical pretreatment methods include acid and alkaline delignification, ammonia recycled percolation or ozonolysis. The most common method is acid pretreatment where acid, often sulfuric acid (H_2SO_4) or hydrochloric acid (HCl), is used to break down the lignin. Acid is usually used at diluted concentration because concentrated acid is toxic, corrosive and hazardous. This is done at high temperatures in order to expedite the breakdown of undesired materials. When acid is used as pretreatment it is necessary to neutralize the pH before enzymatic hydrolysis and/or fermentation process (Sun & Cheng, 2002).

2.4.4.3 Physical pretreatment

Physical pretreatment includes chipping, grinding and milling the biomass to reduce cellulose crystallinity. The energy input of these processes depends on method used and final particle size. Physical pretreatments increase cellulose reactivity towards enzymatic hydrolysis but may be unattractive due to high energy and capital costs (Sánchez & Cardona, 2008). Pyrolysis has been used as a physical method for pretreatment of lignocellulosic biomass. Using this method, materials are treated at temperature higher than 300°C causing the cellulose to rapidly hydrolyse (Sun & Cheng, 2002).

2.4.4.4 Physio-chemical pretreatment

Physio-chemical pretreatments are often more effective and thus more used than physical methods.

Steam-explosion is the most common method used for pretreating lignocellulosic biomass (Sun & Cheng, 2002). Chipped biomass is put in large containers and temperature up to 240°C and pressure (0.69 – 4.83 Mpa) is applied. After few minutes the steam is quickly released and biomass cooled down. This causes the water in the biomass to explode. After the pretreatment the hemicellulose is soluble and the cellulose more accessible for enzymatic hydrolysis (Hendriks & Zeeman, 2009). This method requires low energy compared to mechanical methods (approximately 70% less energy required) and is one of the most costeffective pretreatment for hardwoods and agricultural residues. The disadvantages are that

steam explosion destructs a portion of the xylan fraction and the lignin is sometimes not completely broken down which can have inhibitory effect to microorganisms (Sun & Cheng, 2002).

2.4.5 Enzymatic hydrolysis of cellulose

In order to achieve effective fermentation of lignocellulosic biomass it is necessary to degrade cellulose into glucose and hemicelluloses to monosugars by using acids or enzymes. Enzymatic hydrolysis has shown good results. The utility cost of enzymatic hydrolysis is low compared to acid or alkaline hydrolysis since enzymatic hydrolysis is generally conducted at mild conditions and does not cause corrosion.

The enzymes used are produced by bacteria and fungi that are both aerobic or anaerobic, mesophilic or thermophilic (Sun & Cheng, 2002) and are called *cellulases*. The most studied organisms in cellulase production is the fungi *Trichoderma reesei* that produces three types of cellulases; endoglucanase which randomly attacks β-1,4-glucan chains of cellulose, exoglucanase which releases cellobiose from the non-reducing ends of cellulose chain and cellobiase which hydrolyzes cellobiose to glucose (Lee, 1997; Glazer & Nikado, 2007). Additionally many enzymes degrade hemicellulose. Those enzymes are generally called hemicellulases and are able to break down xylan or different sidechains within the xylan residues (Collins *et al.*, 2005).

Many factors affect enzymatic hydrolysis including substrates, cellulase activity, temperature and pH. One of main factors is substrate concentration. High substrate concentration can cause inhibition, thus, lowering the rate of the hydrolysis (Sun & Cheng, 2002). If substrate concentration is increased it can result in increased reaction rate of the hydrolysis (Cheung & Anderson, 1997). Lignin is closly associated with cellulose microfibrils and can therefore interfere with enzymatic hydrolysis; it can prevent the enzyme attack on cellulose. Presence of hemicellulose in biomass can also cause similar problem and investigations have shown that removal of hemicellulose increases cellulose digestion even though lignin content is high (Zhu *et al.*, 2008).

2.4.6 Fermentation of lignocellulosic biomass

The next step in ethanol and hydrogen production from lignocellulosic biomass after pretreatment is fermentation. A great variety of microorganisms are known ethanol and hydrogen producers as previously described and some of them have been investigated to some extent considering fermentation of hydrolysates.

Saccharomyces cerevisiae and *Zymomonas mobilis* are the most effective ethanol producers known and none of the thermophilic strains described can compete with them considering ethanol yield and tolerance. However, wild types of those two microorganisms are not able to degrade pentoses and are thus not well suited for converting the hemicellulose fraction of lignocellulosic biomass (Sommer *et al.*, 2004).

One example of hydrogen production from hydrolysates is from de Vrije and co-workers (2002), where pretreated *Miscanthus* was used as a substrate for hydrogen production by *Thermotoga elfii*. *Miscanthus* is a woody grass species well suited for biofuel production, giving high yields per hectare. In the experiment done, the biomass was pretreated with mechanical and chemical methods and the enzyme *CelluclastTM* used for further degradation. Growth of *T. elfii* on hydrolysates from *Miscanthus* was compared to growth on glucose supplemented medium. The results showed that hydrogen yields on hydrolysates were slightly higher than on glucose medium. The conversion of biomass into monosaccharides was 62.5% (de Vrije *et al.*, 2002).

Research on ethanol production from hemicellulose has been done by Sommer and co-workers (2004) where wet oxidation was used as a pretreatment on wheat straw. Different concentration of the hydrolysates were tested as substrate for growth for 21 thermophilic strains, giving up to 25.7 mM of ethanol (Sommer *et al.*, 2004).

Generally the hydrolysis and fermentation steps are performed seperately; separate saccharification and fermentation (SHF). The advantages of this method are that each operation can take place at optimum condition, considering e.g. pH and temperature but the accumulation of glucose can have inhibital effect on the enzyme used (Zaldivar *et al.*, 2001). The hydrolysis can also be performed together with the fermentation; simultaneous saccharification and fermentation (SSF) (Öhgren *et al.*, 2007). This method lowers the capital cost (Olofsson *et al.*, 2008) and higher ethanol yields are obtained (Sassner *et al.*, 2008). However, there are also disadvantages using

SSF; a compromise must be found in the operational parameters (pH and temperature), especilly when yeasts are used for the fermentation. The yeast cannot be re-used in SSF and recirculation of enzymes are also difficult (Olofsson *et al.*, 2008).

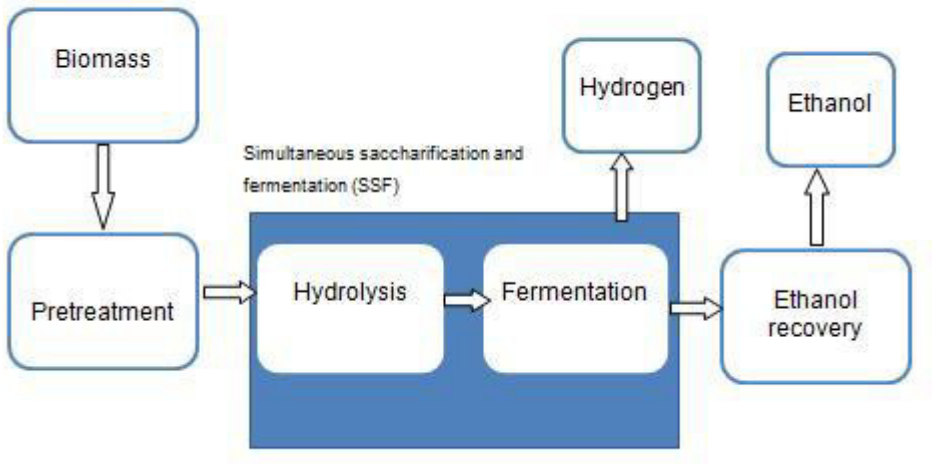

Figure 7. Schematic overview of ethanol and hydrogen production from biomass.

2.4.7 Negative effects of pretreatments

Pretreatment of lignocellulosic biomass can have negative effects because toxic compounds, like phenolic compounds, furan derivatives and weak acids, are generated (Palmqvist & Hahn-Hägerdal, 2000a). This is dependent on both the raw material used and operation conditions used in the hydrolysis. When toxic compounds are generated they affect the metabolism of the fermentative organism used and thus the final ethanol production yield. Those toxic compounds can be divided in four groups: sugar derived products, lignin derived products, compounds derived from lignocellulosic structure and heavy metal ions (Mussatto & Roberto, 2004). Investigations have shown that by removing the hemicellulose, from which those toxic compounds are derived, from the cellulose before enzymatic treatment the toxic effect can be minimized, however lower yields are achieved (Klinke *et al.*, 2004).

2.4.7.1 Sugar derived products

When biomass is hydrolysed, pentose sugars are in some cases degraded to the toxic compound furfural. This can inhibit cells and affect the specific growth rate and cell-

mass yield per ATP as well as specific ethanol productivity (Mussatto & Roberto, 2004; Palmqvist & Hahn-Hägerdal, 2000b). Hydroxymethyl furfural (HMF) is a toxic compound that is formed from hexose degradation. Inhibitory effect of HMF is similar to furfural but is considered to be less toxic. Additionally, the concentration of HMF in hemicelluloses hydrolysates is generally low (Palmqvist & Hahn-Hägerdal, 2000b). Both of these components must be removed or neutralised before fermentation. If not, higher number of fermenting microorganisms must be applied in the fermentation process (Hamelinck *et al.*, 2005).

2.4.7.2 Products derived from lignin and lignocellulosic structure

Phenolic compounds are released from lignin when lignocellulosic biomass is hydrolysed. Those compounds cause damage on biological membrane and affect the ability to serve as selective barriers and enzyme operation (Mussatto & Roberto, 2004; Palmqvist & Hahn-Hägerdal, 2000b).

2.4.7.3 Heavy metal ions

Corrosion of hydrolysis equipment can generate heavy metal ions like iron, nickel and copper. These compounds are toxic in that sense that they inhibit enzymes in metabolic pathways of the microorganism (Mussatto & Roberto, 2004)

2.4.8 Detoxification

Several methods of removal of inhibitors have been proven in order to increase the fermentability of hydrolysates. The method chosen depends on the microorganisms used for the fermentation as well as the raw material. Because detoxification increases the overall cost of the process, it would obviously be better if no such procedures were needed (Olsson & Hahn-Hägerdal, 1996). There are four ways of minimize the presence of inhibitors:

- avoid formation during hydrolysis
- detoxify before fermentation
- develop resistant microorganisms

- convert toxic compounds into others that do not interfere with metabolism.

It is possible to remove the undesired compounds by separating the slurry of pretreated biomass in a liquid and solid fraction. Water is then used to wash the pressed solid fraction to move the inhibitory materials to the liquid fraction. At last, continuous ion exchange and overliming with calcium hydroxide is used on the liquid fraction to form gypsum (Hamelinck *et al.*, 2005).

2.4.9 Improvement of ethanol production from lignocellulosic biomass

Huge amount of lignocellulosic materials are currently burned for heating but its conversion to biofuels could replace 40% of the gasoline in the US market (Wheals *et al.*, 1999). Although recent investigations have improved the production of ethanol from lignocellulosic biomass, it is still not profitable compared to traditional feedstock (Yuan *et al.*, 2008). Complete hydrolysis of cellulose and hemicellulose is required with efficient fermentation of all the sugars in biomass. When the biomass is hydrolysed it contains both hexoses and pentoses and microorganisms that are able to convert many sugars efficiently are neccessary to achieve economical conversion of biomass and high ethanol yield (Becker & Boles, 2003; Hahn-Hägerdal *et al.*, 2006).

The cost of lignocellulosic ethanol is however expected to decrease as the technologies and biomass crops are modified for higher yields and lower resistance to microbes and enzymes (Yuan *et al.*, 2008). It has already been reported that switchgrass is a profitable crop and could even economically be better suited than sorghum and maize (Monti *et al.*, 2007). Improving lignocellulosic ethanol production highly depends on biotechnological research in order to reduce cost and improve processing efficiency (Yuan *et al.*, 2008).

2.5 Factors affecting hydrogen and ethanol production

In order to achieve the highest hydrogen yield it is important to direct the bacterial metabolism towards acetate production. Reduced fermentation end products like ethanol, lactate and butyrate lower the hydrogen yield because they include hydrogen that has not been liberated as gas (Hawkes *et al.*, 2002; Levin *et al.*, 2004).

Factors affecting ethanol and hydrogen production are e.g. pH, temperature, substrate concentration and hydrogen partial pressure (Levin *et al.*, 2004; Ni *et al.*, 2006). In complex systems hydrogen consuming bactera, like methanogens, inhibit hydrogen production. Low pH (< 5.5) can inhibit the growth of methanogens but too low pH inhibits the activity of the hydrogenase which leads to increasing solvent production, e.g. ethanol, acetone and butanol (Li *et al.*, 2007). This has been observed for the bacterium *Clostridium acetobutylicum* which changes from producing hydrogen, acetate and butyrate to the production of acetone and butanol when the pH is decreased to 5.0 or lower (Bahl *et al.*, 1982). The optimal pH for hydrogen production depends on the microorganism but generally varies in the range of 4.0 – 9.0 (Fang & Liu, 2002). Production of acetate and butyrate dominates at pH lower than 5.5 and higher than 6.0 but propionate production increases at pH 5.5 – 6.0. Acetate and butyrate production favours hydrogen production whereas propionate production decreases hydrogen production (Li *et al.*, 2007).

Temperature is one of the most important factor and influences all physiological activity of the microorganism (Li *et al.*, 2007). Hydrogen producing microorganisms have been studied in various temperature ranges from mesophilic to hyperthermophilic. However, thermophilic bacteria have some advantages in both hydrogen and ethanol production: (Kosaric & Vardar-Sukan, 2001; Lee, 1997; Hallenbeck, 2009; Levin *et al*., 2004).

- Higher catabolic activity at optimal temperatures which results in shorter fermentation times, increase in fermentation efficiency and higher productivity.
- Solubility of oxygen and other gases in culture media decreases with higher temperature.

- Less energy is required to maintain proper agitation of growth media since the viscosity of the media decreases at higher temperatures. Additional energy to maintain vessels at desired temperature and cooling requirements after sterilization are less at high temperature.
- Hydrogen production is less affected by partial pressure of hydrogen
- Ethanol recovery is higher at increased temperatures.
- Sterile conditions are not as essential at thermophilic conditions because no obligate thermophilic pathogens are known. Contamination may however occur by thermophilic fungi or other bacteria.

Substrate concentration can play an important role in hydrogen and ethanol production. It has been demonstrated that an increase in substrate concentration could increase hydrogen production but on the other hand, substrate concentration at higher levels would be inhibitory through pH depletion, acid production or increased hydrogen partial pressures (Wang & Wan, 2009; van Ginkel & Sung, 2001). The result can be end product inhibition because of high concentration of organic acids released from the substrates (such as butyrate and acetate) (van Ginkel & Logan, 2005). This has for example been observed for *Citrobacter* strain where glucose concentration was increased (1 – 20 g L^{-1}) in culture media. The result was higher hydrogen production, however, the yield of the production decreased with increased glucose concentration (Oh *et al.*, 2003).

The yield of hydrogen production in dark fermentation is highly dependent on the partial pressure of hydrogen (Hawkes *et al.*, 2002; Levin *et al.*, 2004). It is known that high partial pressure of hydrogen has a negative effect on the production by decreasing the activity of hydrogenase (Kim *et al.*, 2006a). When hydrogen concentration increases the metabolic pathways shift towards producing more reduced end products, e.g. lactate, ethanol or acetone (Nath & Das, 2004) but at higher temperatures the production is less affected by partial pressure (Levin *et al.*, 2004). Thus, the partial pressure of hydrogen in the gas phase should be kept below 2 kPa (Claassen *et al.*, 1999). Gas sparging has shown promising results in reducing partial pressure of hydrogen in the liquid phase and increase the hydrogen yield. Mizuno and

co-workers (2000) reported that the hydrogen production increased from 1.466 mL H_2 min^{-1} g^{-1} biomass to 3.131 mL H_2 min^{-1} g^{-1} biomass under nitrogen sparging conditions (Mizuno *et al.*, 2000). However, the hydrogen concentration in the gas phase is diluted which results in an increase in the cost to recover the hydrogen and is a great disadvantage (Li & Fang, 2007).

Ethanol tolerance of bacteria is a major factor concerning production on industrial scale. Thermophilic bacteria generally have low ethanol tolerance (< 2%). Therefore, more energy is required for ethanol recovery by distillation process than would be if the ethanol concentration is higher in the fermentation media (Georgieva *et al.*, 2007). When the concentration of ethanol increases, impairment of membrane integrity appears for most microorganisms. This is in correlation with the type of lipids in the cellular membrane. *S. cerevisiae* and *Z. mobilis* have different membranes; the former is rich in sterols wheras the latter is rich in the fatty acid *cis*-vaccenic acid and hopanoids. The tolerence to ethanol is different between those two, *S. cerevisiae* tolerates up to 21% but *Z. mobilis* up to 12% (Zaldivar *et al.*, 2001). Ethanol tolerance of several thermophilic bacteria has been increased by cultivating at high ethanol concentration (Georgieva *et al.*, 2007).

3. Research of present study

Present study is based on two earlier projects at the University of Akureyri, supervised by Dr. Jóhann Örlygsson. The former project was based on the study of cellulose degrading bacteria and was called *Energy from paper* (2006). The research objective was to isolate thermophilic bacteria, able to degrade cellulose paper with the main aim to use paper, e.g. newspaper, for ethanol production. Over 60 samples were taken from various hot springs at the geothermal area in Graendalur in South West of Iceland. One of the isolated strains showed closest relation to *Caloramator viterbensis* which is the only thermophilic bacteria known capable of degrading glycerol to the high valuable compound 1,3-propanediol (Seyfried *et al.*, 2002). Glycerol is a by-product in biodiesel production and increased production has led to excessive supply of glycerol on the market. Thus, glycerol is now low priced but is well suited for 1,3-propanediol production.

In June 2007 more samples were taken from the geothermal area in Graendalur. Samples were enriched on different carbon sources, there among glycerol in order to isolate thermophilic strains potential for 1,3-propanediol production from glycerol. Four enrichment cultures were obtained on glycerol. One of them showed the closest relation to *C. viterbensis* and was therefore chosen for further physiological studies. Additionally, one isolate from cellulose showed the closest relation to *C. viterbensis* and was therefore chosen for further physiological studies. Both of those are new species belonging to the genus *Caloramator* and are described in chapter 5 (manuscript I).

The latter project, *Bioethanol*, is based on ethanol production from complex biomass where the aim is to isolate thermophilic bacteria with the ability to degrade lignocellulosic biomass to ethanol. Presently, ethanol production is mostly produced from sugars and starch which has been debated because of its competition with the food and feed market. Thus, lignocellulosic biomass has gained much attention since it cannot be used for food. Another possibility is hydrogen production from lignocellulosic biomass. In Iceland there is a variety of biomass with potential to use

in such production, i.e. agricultural waste and waste paper (newspaper). Earlier investigations have shown that grass could be used for ethanol production (data not shown); therefore broader variety of biomass was tested with the same purpose. One of the strains showed good hydrogen production capacity in batch cultures and was therefore chosen for further analysis on complex biomass. Phylogenetic studies revealed that this strain belongs to the genus *Thermoanaerobacterium* and is closely related to known ethanol and hydrogen producers. The strain is described in details with respect to phylogenetic and physiology in chapter 6 (manuscript II).

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5. Manuscript I

Caloramator ethanolicus sp. nov. and *Caloramator islandicum* sp. nov., novel thermophilic bacteria isolated from Icelandic hot spring

Caloramator islandicum, sp. nov. and *Caloramator ethanolicus, sp. nov.*, novel thermophilic bacteria isolated from Icelandic hot spring

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Abstract

Novel, thermophilic, anaerobic bacteria, strains AK_{44} and AK_{35} were isolated from hot springs in the Hengill area in SW-Iceland. Cells of these organisms were Gram - negative, rod-shaped and motile. The strains were strict anaerobic, capable of growth on various carbohydrates and glycerol. Growth was enhanced in the presence of yeast extract. Growth for strain AK_{44} was observed at pH's between 4.0 to 8.0 and temperatures between 40 to 60 °C; optimal growth conditions were at pH 5.0 and 50.0 °C. Growth for strain AK_{35} was observed at pH's between 6.0 to 8.0 and temperatures between 40 to 60°C; optimal growth conditions were at pH 7.0 and 50°C. Effect of increased substrate concentration was investigated for both strains. Correlation was between substrate loading and end product formation up to 50 mM glucose (AK_{44}) and 100 mM (AK_{35}), then a clear inhibition was observed. The ability to utilize various substrates was tested. The major end product for strain AK_{44} was ethanol; production from glucose was 1.5 mol EtOH per mol glucose. Strain AK_{35} mainly produced acetate and hydrogen. Good carbon recovery was obtained for strain AK_{44} on glucose, fructose and mannose (production from YE subtracted). Strain AK_{35} did not obtain full carbon recovery on any of the substrates tested but the highest recovery was on mannose. Growth experiment was done for both strains using glucose as a sole carbon source. As determined by full 16S rRNA analysis, both strains belong to the genus *Caloramator* with closest relation to *C. viterbensis*. The gene bank accession numbers for the complete 16S rRNA sequences are xxx and xxxx. On the basis of physiological and molecular properties of the new isolates, it is concluded that strains AK_{44} and AK_{35} represent new separate species within the genus *Caloramator*, for which the names *Calaromator ethanolicus* and *Caloramator islandicum* are proposed.

Key words: ethanol production, hot spring, Iceland, *Caloramator ethanolicus*, *Caloramator islandicum*.

Introduction

In 1987, Patel and coworkers described the bacterium *Clostridium fervidus*, isolated from a hot spring in New Zealand. This bacterium is strictly anaerobic, Gram negative, sporeforming and motile; capable of carbohydrate fermentation. The major end product formed is acetate but other end products are produced in minor quantities (Patel *et al.*, 1987). Later, Collins and coworkers reassigned this bacterium to the genus *Caloramator*, based on further phylogenetic studies which showed that *C. fervidus* clearly forms a distinct line at the *Clostridium* genus level (Collins *et al.*, 1994). The *Caloramator* genus consists of seven thermophilic bacteria; *C. fervidus* (Patel *et al*., 1987), *C. coolhaasii* isolated from thermophilic methanogenic granular sludge (Plugge *et al*., 2000), *C. indicus* isolated from an artesian aquifer in India (Chrisostomos *et al*., 1996), *C. proteoclasticus* isolated from granular methanogenic sludge (Tarlera *et al*., 1997), *C. viterbensis* isolated from a hot spring in Italy (Seyfried *et al*., 2002), *C. uzoniensis* (Kevbrin *et al.*, 2002) and *C. australicus*, isolated from the Great Artesian Basin, Australia (Ogg & Patel, 2009).

A general description of the genus is as follows: thermophilic, chemoorganoheterotrophic anaerobic bacteria with a low G+C content. Bacteria belonging to the genus stain Gram – negative with exception of *C. viterbensis* and *C. australicus* (Seyfried *et al.*, 2002; Ogg & Patel, 2009).

Icelandic geothermal areas are potential sources for thermophilic bacteria (Kristjánsson & Alfreðsson, 1986; Sommer *et al.*, 2004; Koskinen *et al.*, 2008). The objective of this study was to isolate thermophilic bacteria, capable of degrading glycerol. Two novel anaerobes were isolated from moderately hot-springs in Graendalur in South-West of Iceland. Their physiology and phylogeny were investigated in detail and revealed that both strains belong to the genus *Caloramator*.

Materials and methods

Growth media. The medium (per liter) consisted of: NH_4Cl 0.3 g, NaCl 0.3 g, $CaCl_2$ 0.11 g, $MgCl_2$ x $6H_2O$ 0.1 g, yeast extract 2.0 g, resarzurine 1 mg, trace element solution 1 ml, vitamin solution 1 ml and $NaHCO_3$ 0.8 g. Phosphate buffers at were also used where 1 M stock solutions of NaH_2PO_4 and Na_2HPO_4 were made and added to the media to give a buffer capacity of 30 mM at the different pH's used. This medium (referred to as BM medium) was used in all growth experiments in this study. Carbon and energy sources were used in 20 mM concentrations or in the case of cellulose, 2 g L^{-1}. The vitamin solution was according to DSM141. The trace element solution was according to Orlygsson & Baldursson (2007). The medium was prepared by adding the buffer to distilled water, which was then boiled for 5-10 min and cooled while flushing with nitrogen. The mixture was then transferred to serum bottles using the Hungate technique (Hungate, 1969) and then autoclaved. Other components of the medium were added separately through filter sterilized solutions. All experiments were carried out in 50 mL serum bottles with 20 mL BM media supplemented with glucose (20mM) unless otherwise indicated. Inoculation volume was 1% and all experiments were done in duplicate.

Isolations of bacteria. Samples were collected from hot springs (56.0°C, pH 5.5 and 50.0°C, pH 7.66 for AK_{44} and AK_{35}, respectively) in Graendalur in the South-West of Iceland by using an extended stick equipped with grip arms placed at the end. Serum bottles (120 mL) were fixed at the end and filled with geothermal mud/liquid samples and closed with butyl rubber and aluminum caps. Five mL of the liquid sample were inoculated into 120 mL serum bottles containing 50 mL of BM medium supplemented with glycerol (100 mM) for AK_{44} and whatman filter paper (99% cellulose) (2 g L^{-1}) for AK_{35}. The samples were incubated at 50°C, slightly below the experimental site temperatures. The pH of the BM medium was 6.0 for AK_{44} and pH 7.0 for AK_{35}.
Because of the dense slurry from the geothermal samples in the beginning it was impossible to follow growth by measuring optical density. Therefore growth was determined by increase in hydrogen production. After five days of cultivation, 5 mL of

the enrichment culture was transferred into a new fresh media. This was repeated three times for both samples and then diluted (tenfold dilution) in the BM medium with the same carbon sources. The last positive sample for each strain was diluted again and inoculated into BM medium with 15 g L^{-1} of BactoAgar in flasks. Carbon source was glycerol (20 mM) for strain AK_{44} and glucose (20 mM) for strain AK_{35}. Visible colonies were picked up with sterile Pasteur pipettes and inoculated into fresh media and re-incubated. This was repeated two times before the two isolates were analyzed for full 16S rRNA sequence analysis. Samples were kept frozen in 30% glycerol until used in all experiments in this study.

Cellular characterization. Examination of purity of cultures and morphology were done using standard phase contrast microscope. Gram-staining was performed using conventional methods (Madigan *et al.*, 2003) and motility was determined using the standard "hanging drop" method. Spore forming was tested by heating cultures at 80°C for 15 minutes and then inoculate into a new fresh media.

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

pH and temperature range determinations. To determine the strain's optimum pH for growth the pH was set to various levels in the range of 3.0 to 9.0 with increments of 1.0 pH unit. The experimental bottles were prepared as before and supplemented with acid (HCl) or base (NaOH) to adjust pH accordingly. For the pH optimum determination the isolates were grown at 50.0°C. For the temperature optimum determination the isolates were grown at pH 5.0 and pH 7.0 for strain AK_{44} and AK_{35}, respectively and at temperature spectrum from 20.0 – 75.0°C. Optimal pH and temperature were thereafter used in all experiments done for both strains.

Effect of glucose concentration and ethanol tolerance. Effect of increased glucose concentration and ethanol tolerance was tested on both strains. Glucose was in different initial concentrations, varying from 5 to 400 mM. Control samples did not contain glucose. Optical density was measured at beginning and at the end of incubation time (5 days) to determine growth. Hydrogen, volatile fatty acids (VFA) and ethanol production was measured as well. Glucose was measured at the end of the incubation time.

Minimum inhibitory concentration (MIC) experiments were carried out to determine the ethanol tolerance for both strains. The ethanol concentration varied from 0 to 8% (vol/vol) and initial glucose concentration was 20 mM. Control samples did not contain glucose or ethanol. Optical density was measured at the beginning and the end of incubation time (5 days) to determine the MIC for the strains.

Substrate utilization. The ability of both strains to utilize various substrates was tested using medium supplemented with various filter sterilized substrates in the final concentration of 20 mM or 2 g L^{-1}. Growth was observed by increase in optical density (600 nm) which was measured at the beginning and at the end of incubation time (five days). Where growth was detected, hydrogen, VFA and ethanol were analyzed.

Growth on thiosulfate was tested to determine the strain's ability to reduce thiosulfate to sulphur or sulphide. Sodium thiosulfate (20 mM) was added to the media but control sample did not contain glucose or thiosulfate. Optical density was measured at the end of incubation time (5 days) to determine growth.

Growth kinetic experiments were done for both strains using glucose (20 mM) as the sole carbon source. Growth was measured on time and end product analyzed to determine generation times and the end product formation.

Analytical techniques. Hydrogen and volatile fatty acids were analyzed with Perkin Elmer gas chromatograph (GC). Hydrogen was measured by manually injecting 200 µL of headspace gas from the culture serum bottles. Micro-thermoconductivity

detector (TCD) was used for hydrogen analysis with nitrogen as the carrier gas (rate 15 mL min^{-1} with another 5 mL min^{-1} as make-up gas in the detectors). The column used was Supelco Carboxen GC Plot Capillary Column (Carboxen 1010) from Supelco. The temperature in both injector and detector was 220°C and the oven was kept at 80°C. Volatile fatty acids and ethanol were analyzed with the same GC but flame ionization detector (FID) and auto injector used. Samples were collected from culture serum bottles and the bacteria removed by filterization through 45 μm Whatman filters. The supernatants (200 μL) were mixed with 100 μL of formic acid, 100 μL of crotonic acid and 600 μL distilled water and put in Perkin-Elmer vials, closed with rubber and aluminium caps. The column used was DB-FFAP from Agilent Industries (Palo Alto, CA, US). The oven temperature started at 80°C and was kept at that temperature for 1 min. before increasing to 160°C over a period of 8 min. This temperature was then kept for 10 min. Determination of glycerol, glucose and 1,3-propanediol was performed by using Shimadzu high performance liquid chromatography (HPLC). The liquid sample (200 μL) was mixed with 100 μL of i-valeric acid and 700 μL distilled water. The HPLC was equipped with Bio-Rad Aminex column (HPX-87H). The mobile phase was distilled water at a flow rate of 0.5 mL min^{-1}. Samples (10 μL) were manually injected to the HPLC using Hewlett Packard syringe. Glucose was also analyzed by slight modification of method from Laurentin and Edwards (2003). Liquid sample (400 μL) was mixed with 2 mL of anthrone solution (0.2% anthrone in 72% sulphuric acid). The sample was boiled for 11 minutes and then cooled down on ice. Optical density was measured at 640 nm.

G + C content. Will be performed by DSMZ.

16S rRNA sequence analysis. For 16S rRNA analysis 16S rRNA genes were amplified from DNA with primers F9 and R1544, specific for bacterial genes (Skirnisdottir *et al.*, 2000) with PCR. The PCR products were sequenced with universal 16S rRNA primers: F9, F515, F1392, R357, F1195 and R1544 by using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Subsequently the DNA was analyzed with 3730 DNA analyzer from Applied Biosystems. The nucleotide sequence was displayed and analyzed with Sequencer

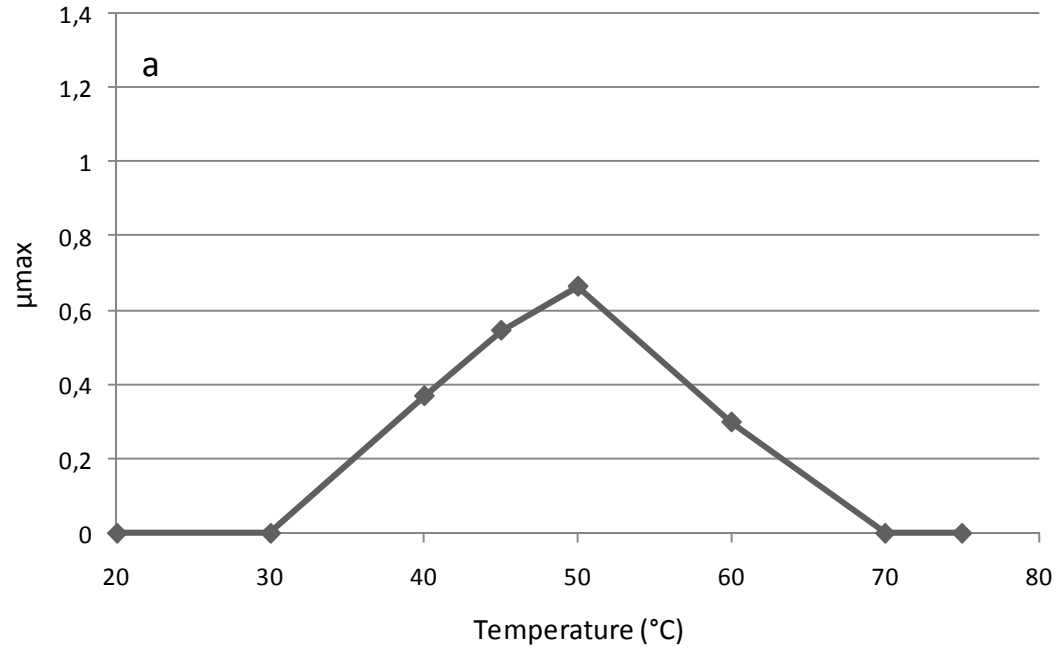
(Gene Code Corporation) (Skirnisdottir *et al.*, 2000). Sequences from 16S rRNA gene analysis were uploaded to the NCBI database using the nucleotide-nucleotide BLAST (BLASTn). Ribosomal Database Project was also used to obtain sequences of related strains. The most similar sequences obtained from the databases were aligned with the results from the sequencing in the program BioEdit and ClustalX where final alignments were done to generate phylogenetic trees. The program TreeCon was used to view the trees. *Escherichia coli* (AE000406) was selected as out-group.

Results

Enrichment and isolation of strains. After repeated enrichment experiments and three end point dilution series (tenfold dilution) with glycerol as carbon substrate, a pure culture of strain AK_{44} was obtained. Strain AK_{35} was isolated with cellulose as carbon substrate by repeated enrichment experiments and three end point dilution series. Colonies were obtained after incubation (one week) into solidified BM medium on glycerol (AK_{44}) or glucose (AK_{35}) as carbon source. The colonies for both strains were round, yellowish and 1.0 to 1.3 mm in diameter.

Morphological characteristics. Cells of strains AK_{44} and AK_{35} were Gram – negative, motile, straight rods, occurring singly or in pairs. Spores were not detected under any of the growth conditions tested.

Temperatures and pH ranges. The temperature growth range for strain AK_{44} was 40.0 to 60.0°C, the optimum being 50.0°C with maximum growth rate (μ_{max}) of 0.66 h^{-1} and doubling time 1.04 h. No growth was observed above 60.0°C or below 30.0°C (Fig. 1a). The pH optimum was 5.0. Below pH 4.0 and above pH 8.0 no growth occurred (Fig. 1b). The maximum growth rate at pH 5.0 was 1.32 and doubling time 0.53 h. The temperature range for strain AK_{35} was 40.0 to 65.0°C with optimum at 50.0°C (Fig. 1c). The maximum growth rate was 0.54 h^{-1} and doubling time 1.27 h. Above 65.0°C and below 40.0°C no growth was detected. The strain was able to grow at pH between 6.0 and 8.0 with an optimum at pH 7.0 where the maximum growth rate was 0.22 h^{-1} (doubling time 3.17 h). No growth was observed below pH 5.0 and above pH 8.0 (Fig. 1d).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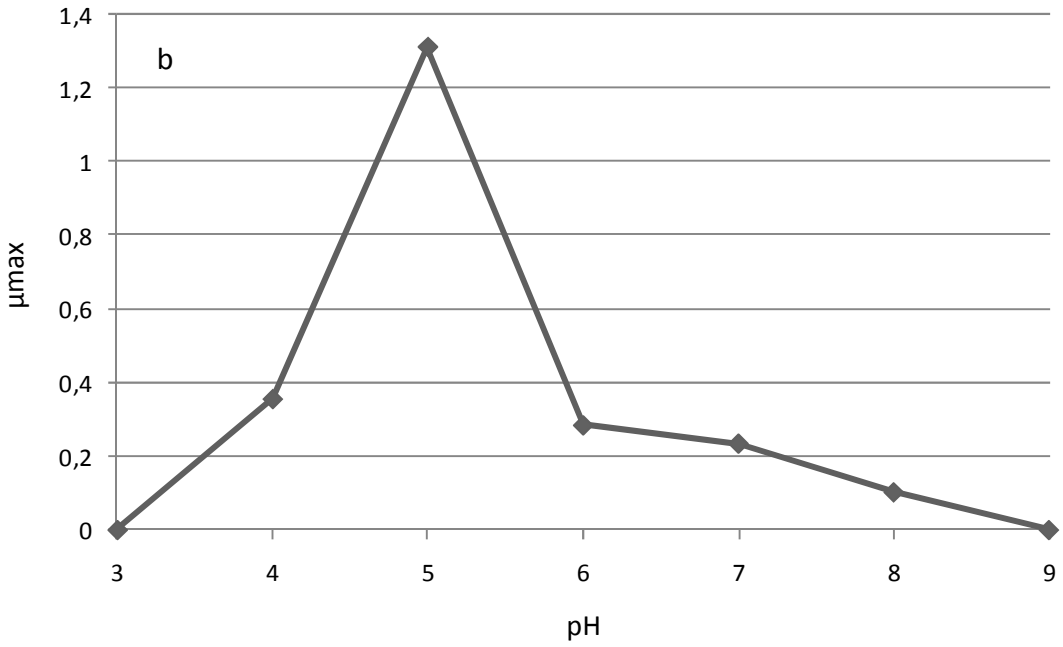
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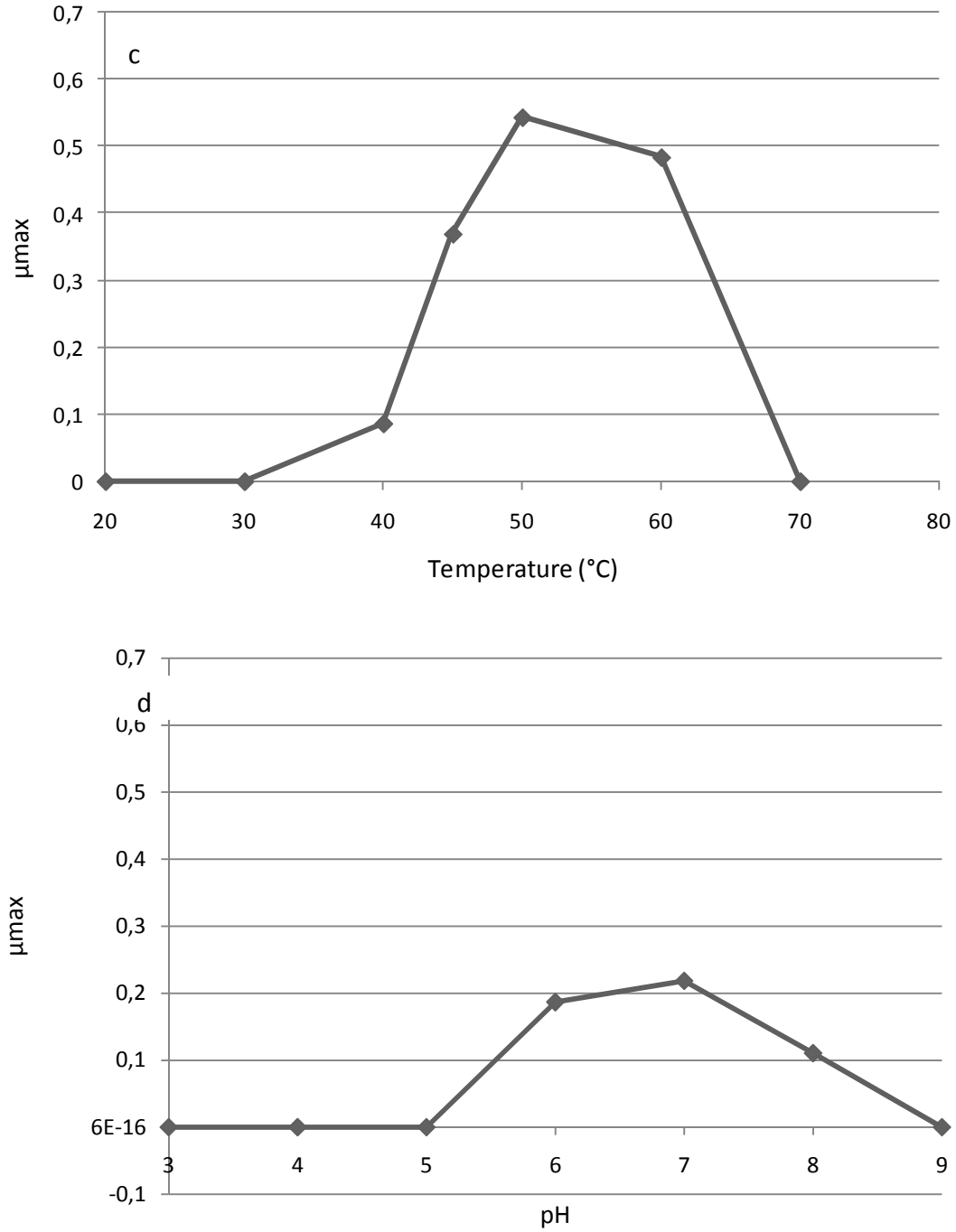
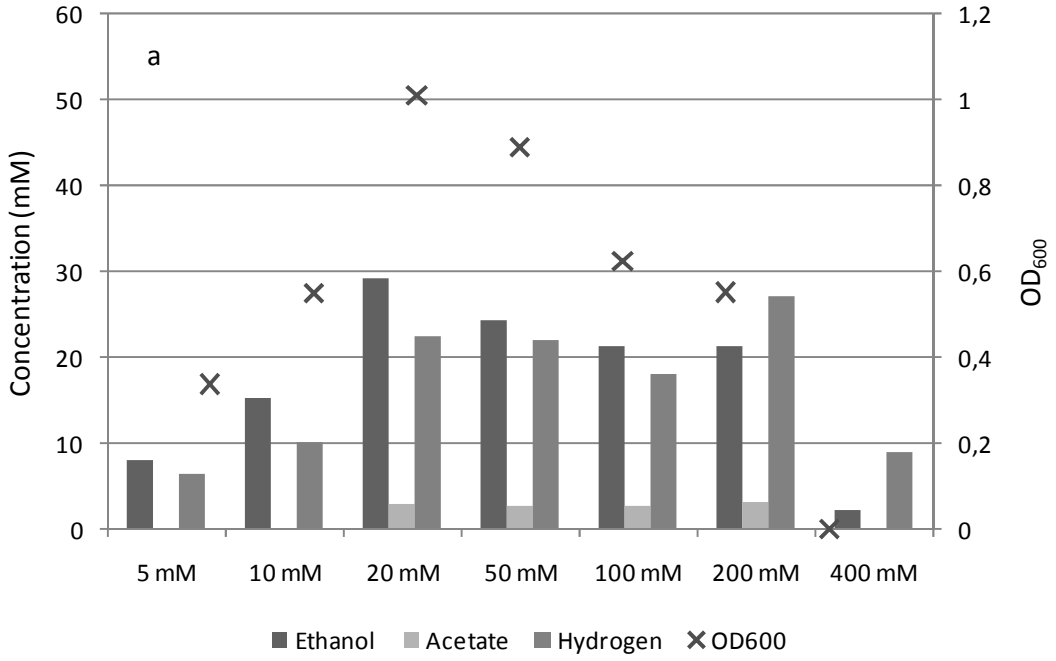


Figure 1. Optimum growth conditions of strains AK_{44} (a) temperature, (b) pH and AK_{35} (c) temperature, (d) pH.

Effect of substrate concentration and ethanol tolerance. Different initial glucose concentration was used to investigate the effect of substrate concentration on growth and end product formation for both strains. It is clear that by increasing initial glucose

concentrations from 5 to 20 mM a good correlation is observed between the initial glucose concentration and the ethanol production for strain AK_{44}, the production is approximately 1.5 mol-EtOH mol-glucose^{-1}. Acetate was only a minor product but relatively high background production (6.2 mM) were produced from YE and has been subtracted from the data shown (Fig. 2a). At increased glucose loadings, a clear inhibition is observed. This can be seen by lower amounts of end product formation (Fig. 2a) as well as partial glucose degradation (Table 1). Strain AK_{35} is more tolerant towards increased substrate loadings (Fig. 2b). End product formation increases in correlation with increased initial glucose concentration. At 100 mM an inhibition in observed which can be seen by slightly lower end product formation as well as partial glucose degradation (Table 1).



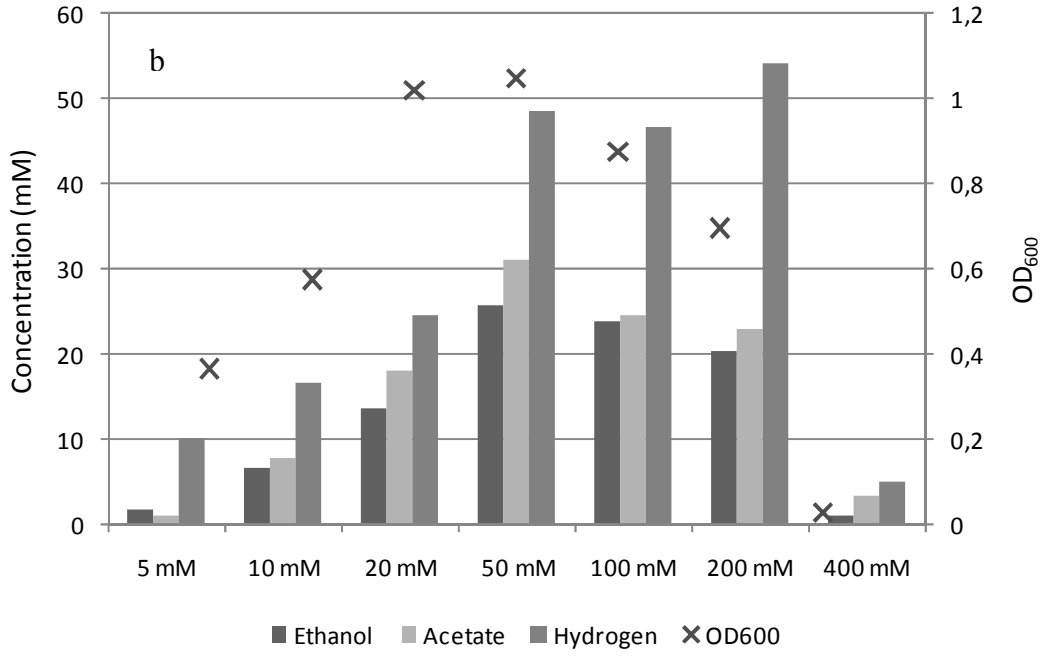


Figure 2. End product formation and growth from different initial glucose concentration for strains AK_{44} (a) and AK_{35} (b).

Table 1. Glucose concentration and carbon recovery for strain AK44. N.c = not calculated.

	Strain AK44		Strain AK35	
Initial glucose concentration (mM)	Glucose concentration after fermentation (mM)	Carbon recovery (%)	Glucose concentration after fermentation (mM)	Carbon recovery (%)
5	0.6	91.8	0.3	30.8
10	0.8	83.3	0.3	75.3
20	1.4	87.0	0.4	81.1
50	41.3	N.c	3.3	60.8
100	97.8	N.c	60.6	61.8
200	197.6	N.c	189.6	N.c
400	399.7	N.c	396.9	N.c

Due to high initial glucose concentration it was difficult to measure the glucose concentration after fermentation which might explain the small amount of fermented glucose compared to end products produced.

Strain AK_{44} showed ethanol tolerance up to 3.2% (vol/vol) and strain AK_{35} up to 3.0%.

Substrate utilization. Of the 36 carbon substrates tested, strain AK_{44} utilized 13 but strain AK_{35} 12 (Table 2). Data for other *Caloramator* strains were obtained from Chrisostomos *et al*. (1996), Plugge *et al*. (2000), Seyfried *et al.* (2002) and Tarlera *et al.* (1997)

Table 2. Utilization of various substrates by AK_{44}, AK_{35}, C. viterbensis, C. indicus, C. proteoclasticus and C. coolhaasii. Nd = not determined, + = growth, - = no growth, (+) = weak growth.

	C. viterbensis (DSM 13723)	*C. indicus* (ACM 3982)	*C. proteoclasticus* (DSM 10124)	*C. coolhaasii* (DSM 12679)	Strain AK_{44}	Strain AK_{35}
Optimum pH	6.0 - 6.5	7.5 - 8.1	7.0 - 7.5	7.0 - 7.5	5.0	7.0
Optimum temp. (°C)	58	60 - 65	55	50 - 55	50	50
Motility	-	-	+	-	+	+
G+C content	32%	25.6%	31%	31.7%		
Glucose	+	+	+	+	+	+
Fructose	+	+	+	+	+	+
Galactose	+	Nd	Nd	+	+	+
Mannose	+	+	+	+	+	+
Xylose	-	Nd	-	+	+	+
Ribose	Nd	Nd	Nd	-	+	+
Arabinose	-	Nd	Nd	-	-	(+)
Maltose	Nd	Nd	Nd	+	Nd	Nd
Sucrose	+	+	Nd	+	(+)	(+)
Lactose	+	+	-	-	+	+
Lactate	Nd	Nd	Nd	Nd	-	-
Formate	-	Nd	Nd	Nd	-	-
Succinate	-	Nd	Nd	Nd	-	-
Malate	-	Nd	Nd	Nd	-	-
Pyruvate	Nd	Nd	+	+	+	+
2-oxoglutarate	Nd	Nd	Nd	-	Nd	Nd
Oxalate	Nd	Nd	Nd	-	-	-
Crotonate	Nd	Nd	Nd	Nd	-	-
Glycerol	+	Nd	Nd	Nd	+	-
Inositol	Nd	Nd	Nd	Nd	-	-
Amylopectin	Nd	+	Nd	Nd	Nd	Nd
Starch	Nd	+	Nd	+	-	+
Amylose	Nd	+	Nd	Nd	Nd	Nd
Dextrin	Nd	+	Nd	Nd	Nd	Nd
Cellobiose	Nd	+	+	+	Nd	Nd
Cellulose	Nd	-	-	-	-	(+)
Xylan	Nd	Nd	Nd	Nd	-	(+)
Sorbitol	Nd	Nd	Nd	Nd	-	-
Sorbose	Nd	-	Nd	Nd	Nd	Nd
CMC	Nd	-	Nd	Nd	Nd	Nd
Dextran	Nd	-	Nd	Nd	Nd	Nd
Yeast extract	+	+	Nd	+	+	+
Pectine	Nd	Nd	Nd	Nd	-	(+)
Gelatine	Nd	Nd	+	+	Nd	Nd
Casein	Nd	Nd	+	+	Nd	Nd
Casamino acids	Nd	Nd	+	+	(+)	+
Casitone	Nd	Nd	Nd	+	Nd	Nd
Peptone	Nd	Nd	Nd	+	+	-
Beef extract	Nd	Nd	Nd	Nd	+	+
Tryptone	Nd	Nd	Nd	Nd	+	+
Alanine	Nd	Nd	-	+	-	-
Aspartate	+	Nd	+	+	-	-
Glycine	Nd	Nd	Nd	Nd	+	+
Glutamine	+	Nd	Nd	Nd	Nd	Nd
Glutamate	Nd	Nd	+	+	-	-
Serine	+	Nd	Nd	Nd	-	-
Threonine	+	Nd	Nd	Nd	-	-
Leucine	+	Nd	+	-	Nd	Nd
Valine	+	Nd	Nd	Nd	Nd	Nd
Histidine	+	Nd	Nd	Nd	-	-
Cysteine	Nd	Nd	Nd	Nd	-	-
Methionine	+	Nd	Nd	Nd	Nd	Nd

The main end product from the carbon substrates (mostly sugars) was ethanol for strain AK_{44} (Fig. 3a). The strain grows well on several hexoses (glucose, mannose, galactose and fructose), pentoses (xylose and ribose) as well as on the disaccharide lactose (Fig. 3a). The only sugar tested which did not support growth was the C_5 monosugar, arabinose. The strain also grew well on glycine with acetate as the main product. In general, proteinous complex substrates (tryptone, peptone, beef extract, casamino acids and yeast extract) supported growth for this strain. Pyruvate was also utilized by strain AK_{44} as well as glycerol, which was mainly converted to ethanol but the strain was initially enriched on this substrate.

Strain AK_{35} was similar concerning substrate utilization as compared to strain AK_{44}. This strain was also saccharolytic but the main end products are acetate and hydrogen (Fig. 3b). Additionally, the strain could utilize pyruvate but weak growth was observed on the polysaccharides pectin and cellulose as well as on complex substrates like beef extract and casamino acids. The strain grew well on starch with hydrogen production of 29.7 mmol L^{-1}.

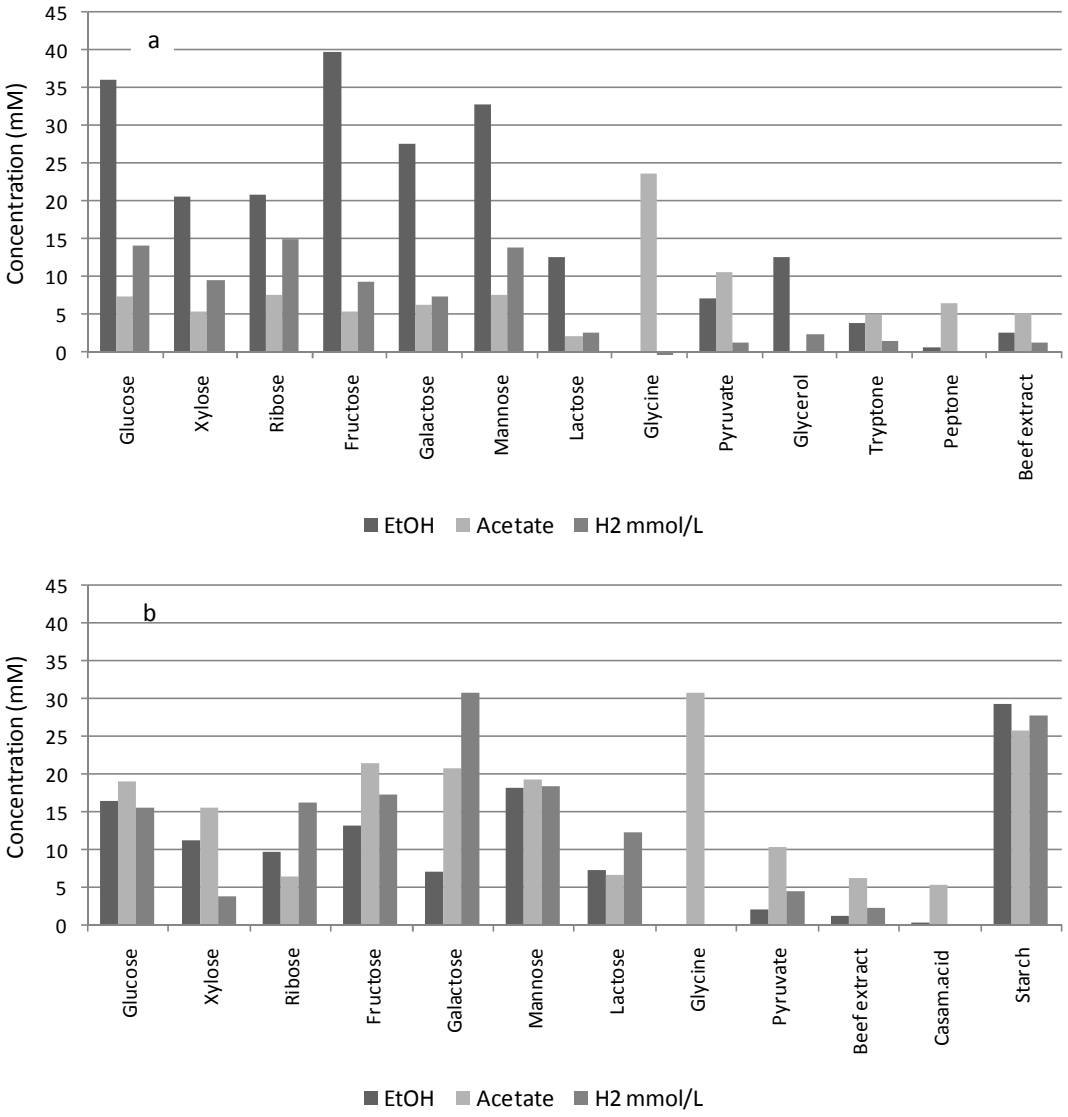


Figure 3. End product formation from different substrates for strain AK_{44} (a) and AK_{35} (b).

Carbon and electron recovery for growth on the saccharides tested were calculated from the data in Figure 3 for both strains (Table 3).

Table 3. Carbon and electron recovery for growth on saccharides for AK_{44} and AK_{35}.

	Carbon recovery (%)		Electron recovery (%)	
Substrate	**Strain AK_{44}**	**Strain AK_{35}**	**Strain AK_{44}**	**Strain AK_{35}**
Glucose	108.3	88.8	103.6	79.3
Xylose	78.1	80.9	59.7	52.3
Ribose	85.7	49.1	67.2	41.9
Fructose	112.9	86.5	105.9	76.8
Galactose	84.9	70.3	78.5	70.7
Mannose	100.8	94.4	96.4	86.0
Lactose	18.4	17.7	16.8	17.9
Sucrose	15.5	5.5	4.3	5.6

More than 100% carbon recovery was obtained for strain AK_{44} on glucose, fructose and mannose but the carbon in biomass that was not measured but is assumed to be 10%. Strain AK_{35} has lower carbon recovery compared to strain AK_{44}, with exception of mannose where almost 100% recovery was calculated. Low carbon recovery was obtained for both strains on lactose and sucrose which is not surprising since small amount of end products is produced using those carbon sources. Electron recovery was in general higher for strain AK_{35} compared AK_{44} with the exception of glucose and fructose where strain AK_{44} achieves more than 100% recovery. Other end products, e.g. lactate, butyrate and formate, were not detected, thus the electron recovery is surprisingly low. One explanation might be excretion of pyruvate or production of succinate, however, this was not measured.

Production of 1,3-propanediol was not detected for neither of the strains.

Neither strain AK_{44} nor AK_{35} were able to utilize thiosulfate.

The kinetics of glucose degradation to various end products was followed. Strain AK_{44} had a doubling time of 2.8 h. The ethanol and hydrogen production rates were 4.1 mM EtOH h^{-1} and 1.5 mmol H_2 L^{-1} h^{-1}. Glucose was completely degraded after approximately 30 hours (Fig. 4a). Strain AK_{35} grew slightly slower compared to strain AK_{44}, the doubling time was 3.2 h. The ethanol, acetate and hydrogen production rates were 2.4 mM EtOH h^{-1}, 2.1 mM Ac h^{-1} and 1.8 mmol H_2 L^{-1} h^{-1}, respectively. The strain degraded glucose completely after 25 hours (Fig. 4b). The results are in good

correlation to what was observed in Fig. 3, i.e., the main end product for strain AK_{44} was ethanol (32.1 mM) but acetic acid for strain AK_{35} (25.9 mM). Ethanol, acetic acid and hydrogen were the only end products detected (Table 4).

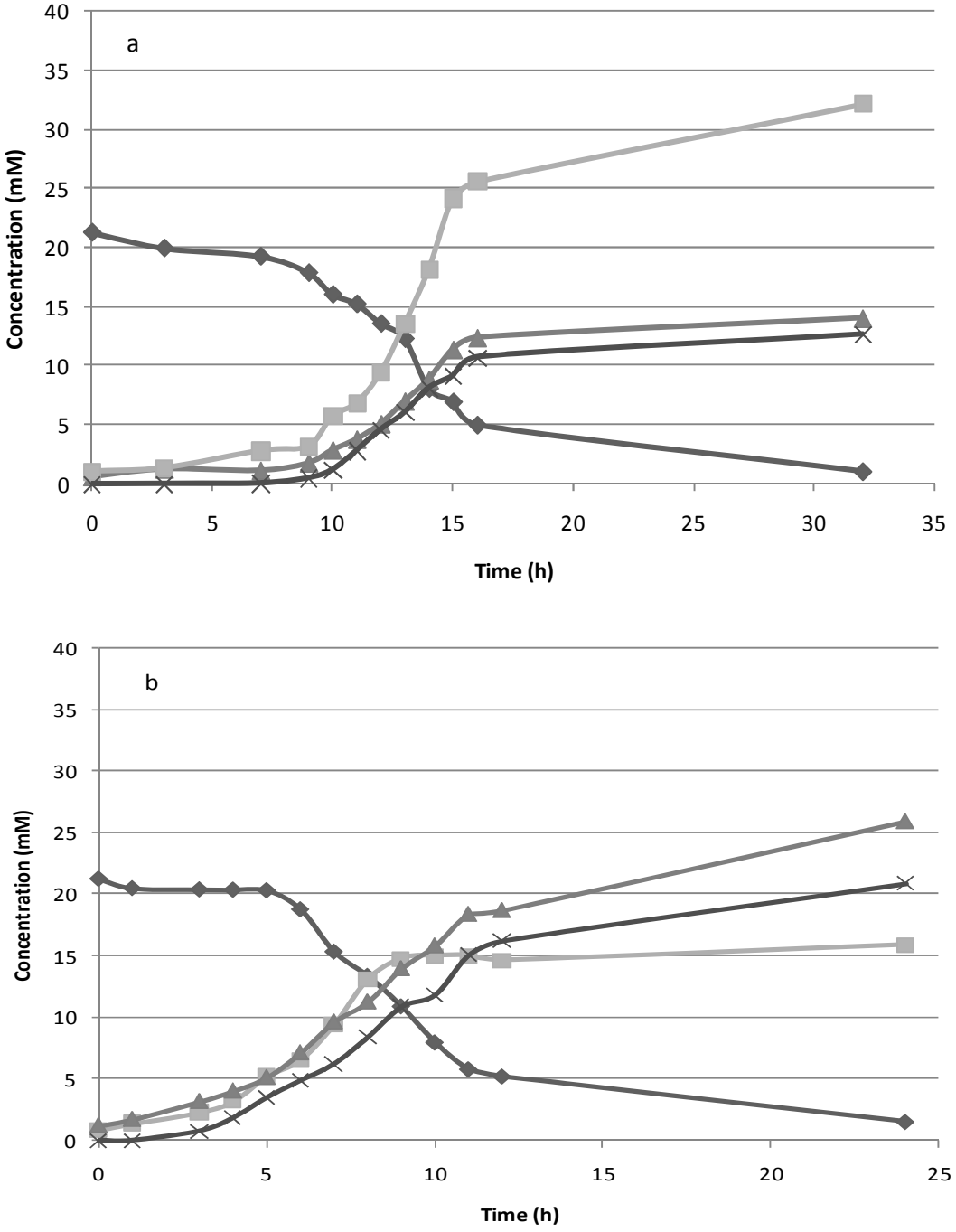


Figure 4. Growth of strains AK_{44} (a) and AK_{35} (b) with glucose as carbon source; ∆= acetate, x = hydrogen, □ = ethanol, ◊ = glucose. Control sample was subtracted.

Table 4. Mole of fermentation products per mol of glucose, carbon and electron recovery.

Strain/mol	Glucose	Ethanol	Acetate	Hydrogen	CO_2	Carbon recovery (%)	Electron recovery (%)
AK_{44}	1.00	1.60	0.70	0.63	2.3	111.18	104.6
AK_{35}	1.00	0.79	1.29	1.04	2.08	104.4	92.5

Table 4 shows the mole to mole production of glucose to end products. Carbon dioxide was not measured but was calculated from other products produced. Carbon and electron recovery for glucose fermentation was calculated and is in a good correlation to the data in Table 3.

Phylogeny. Cell material was submitted for full 16S rRNA analysis which showed that both strains are members of the genus *Caloramator*. The closest relative is *Caloramator viterbensis* (DSM 13723) where the similarity was 94.5% for AK_{44} and 92.1% for AK_{35}. Both strains show different characteristics compared to other members of the genus as well as compared to each other. The relation between the two strains is 96.5% and based on their characteristics and end product formation it is concluded that both strains represent new species within the genus. The strains have been submitted to DSMZ and the DNA base composition will be determined. Figure 5 shows a phylogenetic tree constructed for both strains and their closest relatives.

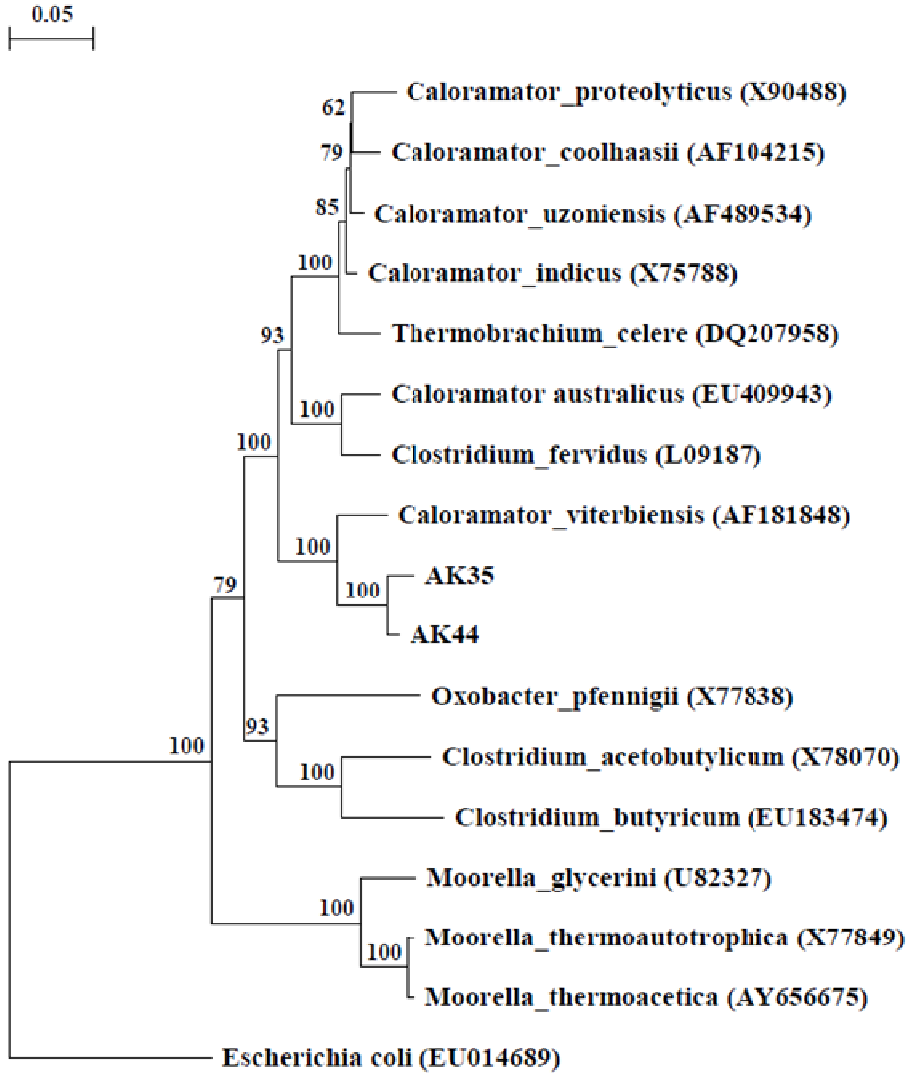


Figure 5. Phylogeny of the strains based on the 16S rRNA gene partial 558 sequences (1450 bp). The phylogenetic tree was generated using a distance matrix and neighbor joining algorithms with 500 bootstraps. Only supported bootstrap values (>50%) are shown. *Escherichia coli* (EU014689) was selected as an out-group. The scale bar indicates 0.05 substitutions per nucleotide position.

Discussion

The novel isolates, AK_{44} and AK_{35}, represent new thermophilic bacteria isolated from Icelandic geothermal areas. Strain AK_{44} was isolated of its ability to ferment glycerol but strain AK_{35} was enriched on cellulose and isolated on glucose. As is apparent from the phylogenetic tree (Fig. 5), both strains are closely related to *Caloramator viterbensis* and four other *Caloramator* species that form a distinct cluster within the Gram-type positive *Bacillus – Clostridium* branch of the phylogenetic tree. Based on full 16S rRNA analysis, strains AK_{44} and AK_{35} have less than 95.0% homology with *Caloramator viterbensis* and should therefore be assigned as new species according to the 96.0% limits set for new species (Stackebrandt *et al.*, 1999).

The optimum temperature for growth of strains AK_{44} and AK_{35} is more similar to *C. proteoliticus* and *C. coolhaasii* than it is to *C. viterbensis* (Table 2). The optimum pH for strain AK_{44} (5.0) is 2 to 3 pH units lower than for other reported strains of the genus except for *C. viterbensis* which has an optimum at pH 6.0 – 6.5. Strain AK_{35} has similar optimum pH (7.0) as other members of the genus (Table 2).

C. viterbensis has been reported to utilize various substrates (Table 2). Unlike strains AK_{44} and AK_{35}, *C. viterbensis* can not grow on xylose. Strains AK_{44} and AK_{35} can not degrade serine, histidine, cysteine and aspartate but *C. viterbensis* can utilize them for growth. *C. viterbensis* is the only thermophilic bacteria reported to be able to convert glycerol to 1.3-propanediol (Seyfried *et al.*, 2002). Strain AK_{44} was able to ferment glycerol to ethanol, however, 1,3-propanediol was not detected. It‘s ability to ferment glycerol is not surprising since it was originally enriched on glycerol. Glycerol did not support growth for strain AK_{35}.

The doubling time for growth under optimum conditions is very similar for both of the isolates compared to the closest relative, *C. viterbensis* although it is several times higher than for the other reported strains. With glucose as the sole carbon source, the doubling time was 2.8 h and 3.2 h for strain AK_{44} and AK_{35}, respectively. The shortest

doubling time for *C. viterbensis* (tested on glycerol) is 2.8 h. However, there is a striking difference in the doubling time between experiments for strain AK_{44} and AK_{35}. The shortest doubling time for strain AK_{44} was obtained when testing the optimal pH for growth (30 minutes) but during experiments on optimal temperature for the strain the doubling time was 1.03 h. The same was obtained for strain AK_{35}, the shortest doubling time was 1.2 h (temperature optimum experiment) but 3.2 h during pH_{opt} experiments. The shortest doubling time was not obtained again for neither of the strain under any condition tested. Further analysis on growth condition considering e.g. the growth media or substrate used, might be needed in order to explain this. As can be seen on Figure 4b the growth curve for strain AK_{35} is almost linear so these growth conditions are not suited for optimum growth of this strain.

Ethanol and hydrogen production among thermophilic bacteria is well known and has been investigated the last few years in terms of possible future renewable energy source (Sommer *et al.*, 2004; Dien *et al.*, 2003). The highest ethanol yield from glucose reported is from the thermophilic bacteria *Thermoanaerobacterium ethanolicus* which produces 1.9 mM-EtOH mol-glucose^{-1} utilized in batch cultures (Kosaric & Vardar-Sukan, 2001). The highest theoretical ethanol yield from glucose is 2 mol mol^{-1} (Olsson & Hahn-Hägerdal, 1996). In present study strain AK_{44} produced 32 mM of ethanol from 20 mM glucose giving the following stochiometry:

1.0 Glucose → 1.6 EtOH + 0.7 Acetate + 0.6 H_2 + 2.3 CO_2

Thus, the strain is clearly a good ethanol producer with 80% of theoretical yield. This characteristic distinguishes strain AK_{44} from other members of the genus *Caloramator* where strains have not been reported for high ethanol production. The main fermentation products for strain AK_{35} are acetate and hydrogen. Theoretically, the maximum hydrogen yield is 4 mol hydrogen mol glucose^{-1} and is achieved when acetate is the main soluble product (Levin *et al.*, 2004). In present study, strain AK_{35} produced 20.8 mM of hydrogen from 20 mM glucose giving the following stochiometry:

1.0 Glucose → 0.8 EtOH + 1.3 Acetate + 1.04 H_2 + 2.08 CO_2

Thus, the strain is producing 26% of theoretical yield of hydrogen.

High initial substrate concentrations are an important factor concerning bioethanol and biohydrogen production from complex biomass (Sommer *et al.*, 2004). Another important factor is the ethanol concentration; the energy demand rises highly if the ethanol concentration is below 2%. Higher substrate concentration thus gives higher final ethanol concentration and is preferred. However, fermenting organisms, especially those who break down sugar polymers, have limited tolerance either towards substrate loadings or ethanol, possibly to both (Olsson & Hahn-Hägerdal, 1996). When ethanol concentration in the broth increases, impairment of membrane integrity is observed for most microorganisms (Zaldivar *et al.*, 2001). When substrate concentration was increased from 20 to 50 mM a clear inhibition was observed for strain AK_{44}. This was shown by lower amount of end products produced (Fig. 2a) as well as insufficient glucose degradation (Table 1). Strain AK_{35} was less affected by increased substrate loading (Fig. 2b) and inhibition was observed at higher than 50 mM glucose concentration. Production of hydrogen was high at 50 to 200 mM although ethanol and acetate production was less than at 20 mM. This can also be seen by the glucose degradation shown in Table 1, whereas strain AK_{35} is degrading up to 40 mM when the initial concentration is 100 mM. However, full carbon recovery is not achieved for neither of the strains.

For an efficient bioethanol and biohydrogen production from lignocellulosic material it is important to use microorganisms that can degrade a broad range of carbohydrates. The main sugars that are released when biomass is hydrolysed are glucose and xylose (Hamelinck *et al.*, 2005). Both strains grow well on those sugars as well as the other tested in this study with an exception of the pentose, arabinose. Thus, it can be concluded that strain AK_{44} would be well suited for bioethanol production from lignocellulosic biomass.

Description of *Caloramator ethanolicus* sp. nov.

Cells of *Caloramator ethanolicus* (AK_{44}) are moderately thermophilic, Gram negative, motile rods occurring as single cells or in pairs. Growth was only observed under anaerobic conditions. Growth occurred with glucose, fructose, galactose, mannose, xylose, ribose, lactose, glycerol, pyruvate, peptone, beef extract, tryptone and glycine as carbon sources. No growth was observed when arabinose, lactate, formate, succinate, malate, oxalate, crotonate, cellulose, sorbitol, pectin, xylan, aspartate, glutamate, serine, histidine, cystein and starch were used as a sole carbon source. Only weak growth occurred on sucrose, inositol, casamino acids and alanine. The optimum temperature for growth is 50.0°C. The maximum temperature for growth is 60.0°C and no growth is observed below 40.0°C. The pH range for growth is 4.0 – 8.0 with an optimum at pH 5.0. Fermentation of glucose yields ethanol and acetic acid as the only organic products. Little amount of hydrogen is produced during growth.

The strain was isolated from water/mud sample taken from an off flow from a hot spring in Graendalur in SW-Iceland. The strain belongs to the genus *Caloramator*. The type strain is XXX (DSM).

Description of *Caloramator islandicus* sp. nov.

Cells of *Caloramator islandicus* (AK_{35}) are moderately thermophilic, Gram negative, motile rods occurring as single cells or in pairs. Growth was observed under anaerobic conditions. Growth is observed with glucose, fructose, galactose, mannose, xylose, ribose, lactose, pyruvate, starch, alanine, glycine, beef extract, tryptone and casamino acids. Only weak growth occurs on arabinose, sucrose, cellulose, xylan and pectin. Succinate, malate, oxalate, crotonate, aspartate, glutamate, lactate, formate, glycerol, inositol, sorbitol, peptone, alanine, serine, threonine, histidine and cysteine are not fermented. Growth is observed at temperature between 40.0 to 60.0°C, the optimum being 50.0°C. The pH range for growth is 6.0 – 8.0 with and optimum at pH 7.0.
Fermentation of glucose yields acetate and hydrogen as the main fermentation products but little amount of ethanol is produced.
The strain was isolated from a water sample taken from a hot spring in Graendalur in SW-Iceland. The strain belongs to the genus *Caloramator*. The type strain is XXX (DSM).

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6. Manuscript II

Hydrogen and ethanol production from sugars and complex biomass by *Thermoanaerobacterium* species AK_{54}, isolated from Icelandic hot spring

Hydrogen and ethanol production from sugars and complex biomass by *Thermoanaerobacterium* species, AK_{54}, isolated from Icelandic hot spring

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Abstract

Strain AK_{54}, a thermophilic ethanol and hydrogen producing bacteria was isolated from hot spring in the Hengill area in SW–Iceland. The strain was anaerobic, capable of growth on mono- and disaccharides. Growth was enhanced in the presence of yeast extract. Growth for strain AK_{54} was observed at pH's between 4.0 to 6.0 and temperatures between 55.0 to 70.0°C; optimal growth conditions were at pH 5.0 and 65°C. Effect of increased substrate concentration was investigated and good correlation observed between substrate loading and end product formation up to 50 mM where clear inhibition was shown. The ability to utilize various substrates was tested. The major end products were ethanol, acetate and hydrogen. Growth experiment was done for strain AK_{54} with glucose as a sole carbon source. Ethanol and hydrogen production from complex biomass was investigated. As determined by full 16S rRNA analysis, strain AK_{54} belongs to the genus *Thermoanaerobacterium* with closest relation to *T. acidotolerans*.

Key words: ethanol, hydrogen, hot spring, *Thermoanaerobacterium*, hydrolysates.

Introduction

Today, most of the world's energy demands are met by non-renewable energy sources, causing depletion, environmental deterioration and public health problems. Energy consumption is growing at rising rates at the same time, demanding novel renewable energy sources (Sánchez & Cardona, 2008). Hydrogen has a great potential as a clean, renewable energy carrier since it is a clean fuel with no CO_2 emissions when combusted (Levin *et al.*, 2004; Kapdan & Kargi, 2006). Additionally molecular hydrogen has high energy yield (122 kJ/g) which is 2.75 times higher than most hydrocarbon fuels (Kapdan & Kargi, 2006). Bioethanol, produced from biomass, has gained increased interest in recent years and is already produced in scale of 51.000 million liters worldwide (Lin & Tanaka, 2006; Sánchez & Cardona, 2008). Many countries are now implementing or have implemented programs for addition of ethanol to gasoline, e.g. in Brazil where ethanol is blended 24% (v/v) in gasoline and 10 – 30% in USA. These two countries are the largest ethanol producers worldwide today with 87% of global production (Sánchez & Cardona, 2008). Currently, ethanol production in Brazil, India and South Africa is mainly based on sugar cane but in USA from corn (first generation ethanol) (Zaldivar *et al.*, 2001). This production has been strongly debated the last few years because it is competing with the food and feed application, leading to increased interest in more complex biomass. Fermentation from lignocellulosic biomass (e.g. wood, straw and grasses) is therefore an interesting alternative for the production of second generation bioethanol and biohydrogen (Balat *et al.*, 2008). Both hydrogen and ethanol can be produced microbiologically through fermentation from various starch- and sugar-based materials (Hawkes *et al.*, 2002), there among lignocellulosic biomass. Attention in hydrogen production through dark fermentation has increased in the past few years and high production rates has been achieved with bacterial fermentations (Wu *et al.*, 2006; Koskinen *et al.*, 2008a; Koskinen *et al.*, 2008b). Thermophiles have many advantages compared to mesophilic microorganisms in hydrogen and ethanol production concerning fast growth rates and their ability to degrade a broad variety of substrates, as well as higher hydrogen yields

(Sommer *et al.*, 2004; van Groenestijn *et al.*, 2002). Furthermore many thermophiles have narrower spectrum of fermentation end products compared to mesophiles.
Hot springs are a potential source for hydrogen and ethanol producing microorganisms (Koskinen *et al.*, 2008a). In this study a thermophilic fermentative bacterium efficient in hydrogen and ethanol production is studied. Hydrolysates from various complex biomass were used to test the performance of the bacterium. Optimal conditions for hydrogen production in terms of temperature and pH were investigated. Kinetic parameters from glucose degradation were identified.

Materials and methods

Media. The medium (per liter) consisted of: NH_4Cl 0.3 g, NaCl 0.3 g, $CaCl_2$ 0.11 g, $MgCl_2$ x $6H_2O$ 0.1 g, yeast extract 2.0 g, resarzurine 1 mg, trace element solution 1 ml, vitamin solution 1 ml and $NaHCO_3$ 0.8 g. Phosphate buffers at were also used where 1 M stock solutions of NaH_2PO_4 and Na_2HPO_4 were made and added to the media to give a buffer capacity of 30 mM at the different pH's used. This medium (hereafter referred to as BM medium) was used in all experiments. The vitamin solution was according to DSM141. The trace element solution used is according to Orlygsson and Baldursson (2007). The medium was prepared by adding the buffer to distilled water, which was then boiled for 5-10 min and cooled while flushing with nitrogen. The mixture was then transferred to serum bottles using the Hungate technique (Hungate, 1969) and then autoclaved. Other components of the medium were added separately through filter sterilized solutions. All experiments done in this study were carried out in 50 mL serum bottles with 20 mL BM media supplemented with glucose (20mM) unless other indicated. Inoculation was 1% and all experiments were done in duplicate.

Isolation of the bacterial strain. Samples were collected from the geothermal area at Graendalur in the South-West Iceland by using an extended wood stick equipped with grip arms placed at the end. Serum bottles (120 mL) were fixed at the end and filled with geothermal liquid. The temperature in the hot spring, from where strain AK_{54} was isolated, was 66°C (measured at sampling site) and pH 5.3 (measured at laboratory).

Five mL of the liquid sample were inoculated into 120 mL serum bottles containing 45 mL BM medium supplemented with glucose (20 mM). The sample was incubated at 60.0°C and pH 6.0.

Because of the dense slurry from the geothermal samples in the beginning it was impossible to follow growth by measuring optical density. Therefore growth was determined by increase in hydrogen production. After five days of cultivation, 5 mL of the enrichment culture was transferred into a new fresh media. This was repeated three times and from the final enrichment culture tenfold dilution were done. End point dilutions were repeated three times before the isolate was analyzed for full 16S rRNA sequence analysis. Samples were kept frozen in 30% glycerol until used in all experiments done in this study.

Strain identification. For 16S rRNA analysis 16S rRNA genes were amplified from DNA with primers F9 and R1544, specific for bacterial genes (Skirnisdottir *et al.*, 2000) with PCR. The PCR products were sequenced with universal 16S rRNA primers: F9, F515, F1392, R357, F1195 and R1544 by using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Subsequently the DNA was analyzed with 3730 DNA analyzer from Applied Biosystems. The nucleotide sequence was displayed and analyzed with Sequencer (Gene Code Corporation) (Skirnisdottir *et al.,* 2000). Sequences from 16S rRNA gene analysis were uploaded to the NCBI database using the nucleotide-nucleotide BLAST (BLASTn). Ribosomal Database Project was also used to obtain sequences of related strains. The most similar sequences obtained from the databases were aligned with the results from the sequencing in the program BioEdit and ClustalX where final alignments were done to generate phylogenetic trees. The program TreeCon was used to view the trees. *Caloramator viterbensisi* (AF181848) was selected as out-group.

Determination of growth. Cell concentration was determined by measuring absorbance at 600 nm by Perkin Elmer spectrophotometer. Maximum (specific) growth rate (μ_{max}) for each growth experiment was derived from the absorbance data (OD_{600}) using the equation: $\ln(x/x_0) = (\mu)(t)$, where x is the measurement of optical

density of the culture, x_0 is the initial optical density of the culture, t is the elapsed time and μ denotes the maximum growth rate.

Determination of pH_{opt} and T_{opt}. To determine the strain's optimum pH for growth the pH was set to various levels in the range of 3.0 to 9.0 with increments of 1.0 pH unit. The experimental bottles were prepared as before and supplemented with acid (HCl) or base (NaOH) to set the pH accordingly. To determine the optimum temperature for growth the incubation temperature varied from 30°C to 75°C. For the pH optimum determination the strain was cultivated at 60°C and for the temperature optimum determination the pH was 5. Control samples did not contain glucose. Optimal pH and temperature were thereafter used in all experiments performed.

Effect of substrate concentration. Effect of increased glucose concentration was tested on strain AK_{54}. Initial glucose concentration varied between 5 to 400 mM. Control samples did not contain glucose. Optical density was measured at beginning and at the end of incubation time (5 days) to determine growth. Hydrogen, volatile fatty acids (VFA) and ethanol were measured as well. Glucose was measured at the end of the incubation time.

Substrate utilization. The ability of strain AK_{54} to utilize different substrates was tested using the BM medium supplemented with various filter sterilized substrates (20 mM or 2 g L^{-1}). Substrates tested were glucose, fructose, galactose, mannose, xylose, ribose, arabinose, sucrose, lactose, lactate, formate, succinate, malate, pyruvate, oxalate, crotonate, glycerol, inositol, starch, cellulose, xylan, sorbitol, pectin, casamino acids, peptone, beef extract, tryptone, alanine, aspartate, glycine, glutamate, serine, theronine, histidine and cysteine. Growth was observed by increase in optical density which was measured at the beginning and at the end of incubation time (5 days). Where growth was detected, hydrogen, volatile fatty acids and ethanol were analyzed.

Growth kinetic experiment was done for the strain using glucose (20 mM) as the sole carbon source. Growth was measured on time and end product analyzed to determine generation time and the production of end products.

Effect of gas-liquid volume ratio on hydrogen production. The influence of partial pressure of hydrogen (ρH_2) on hydrogen production was investigated with different ratios of liquid and gas phase. The liquid phase varied from 2 – 90 mL in serum bottles with total volume of 120 mL, thus, the liquid/gas volume ratio varied from 0.016 to 0.75. After 5 days of incubation, optical density was measured as well as end product formation (hydrogen production, acetate and ethanol). Glucose was measured at the end of incubation time.

Pretreatment of biomass and hydrolysate preparation. Hydrolysates (HL) were made from different biomass: Whatman filter paper (cellulose), hemp (*Cannabis Sativa L.*) – leafs and stem fibers (*Fedora cultivar*), newspaper with ink (NPi), barley straw (BS) (*Hordeum vulgare*) and grass (*Phleum pratense*). The Whatman paper consists of 99% cellulose and was therefore used as control sample. The hemp was collected fresh after harvesting. The hemp, the grass and the barley straw were dried overnight at 50°C and cut into small pieces (< 3 mm). The Whatman paper and newspaper were shredded and then cut with scissors into small pieces (< 3 mm). Ten grams of each type of biomass were weighed into separate Waring blenders and distilled water added until total mass was 400 g, giving final dry weight of 25 g L^{-1}. This was mixed together until homogenized. Thereafter, each mixture was transferred to 400 mL flasks and pretreated by 0.75% H_2SO_4 and 0.75% NaOH (control was without chemical pretreatment) before autoclaving for 30 minutes (121°C). After heating, the bottles were cooled down to room temperature and the pH adjusted to 5.0 by adding either HCl or NaOH. Two enzymes were added to each bottle, Celluclast® and Novozymes 188 (1 mL of each; 0.25% vol/vol), and incubated in water bath at 45°C for 68h. After the enzyme treatment the pH was measured again and adjusted to the pH optimum of the strain. The hydrolysates were then filtered into sterile bottles to collect the hydrolysates.

Fermentation of strain AK_{54} was done in 7 mL BM medium in 120 mL serum bottles. The medium was supplemented with 30% of each hydrolysate (total liquid volume 10 mL) but control sample contained no hydrolysate. The concentration of salts, vitamins and trace elements were kept the same as in the BM medium.

Analytical methods. Hydrogen and volatile fatty acids were analyzed with Perkin Elmer gas chromatograph (GC). Hydrogen was measured by manually injecting 200 µL of headspace gas from the culture serum bottles. Micro-thermoconductivity detector (TCD) was used for hydrogen analysis with nitrogen as the carrier gas (rate 15 mL min^{-1} with another 5 mL min^{-1} as make-up gas in the detectors). The column used was Supelco Carboxen GC Plot Capillary Column (Carboxen 1010) from Supelco. The temperature in both injector and detector was 220°C and the oven was kept at 80°C.

Volatile fatty acids and ethanol were analyzed with the same GC but equipped with flame ionization detector (FID) and auto injector. Samples were collected from culture serum bottles and the bacteria removed by filterisation through 45 µm Whatman filters. The supernatants (200 µL) were mixed with 100 µL of formic acid, 100 µL of crotonic acid and 600 µL distilled water and put in Perkin-Elmer vials, closed with rubber and aluminium caps. The column used was DB-FFAP from Agilent Industries (Palo Alto, CA, US). The oven temperature started at 80°C and was kept at that temperature for 1 min. before increasing to 160°C over a period of 8 min. This temperature was then kept for 10 min. Determination of glucose was performed by using Shimadzu high performance liquid chromatography (HPLC). The liquid sample (200 µL) was mixed with 100 µL of i-valeric acid and 700 µL distilled water. The HPLC was equipped with Bio-Rad Aminex column (HPX-87H). The mobile phase was distilled water at a flow rate of 0.5 mL min^{-1}. Samples (10 µL) were manually injected to the HPLC using Hewlett Packard syringe. Glucose was also analyzed by slight modification of the method from Laurentin and Edwards (2003). Liquid sample (400 µL) was mixed with 2 mL of anthrone solution (0.2% anthrone in 72% sulphuric acid). The sample was boiled for 11 minutes and then cooled down on ice. Optical density was then measured at 640 nm.

Results

Isolation of bacterial strain and phenotypic characteristics. After three series of enrichment cultures, with glucose as a carbon source, the culture was diluted (tenfold dilution). End point dilution series were repeated three times before obtaining pure culture. This pure culture was sent for partial 16S rRNA analysis and later for full 16S rRNA analysis. Full 16S rRNA analysis showed that strain AK_{54} is a member of the genus *Thermoanaerobacterium*. The closest relative is *T. aciditolerans* (DSM 16487) with 99% homology. Figure 1 shows a phylogenetic tree constructed for strain AK_{54} and the closest relatives.

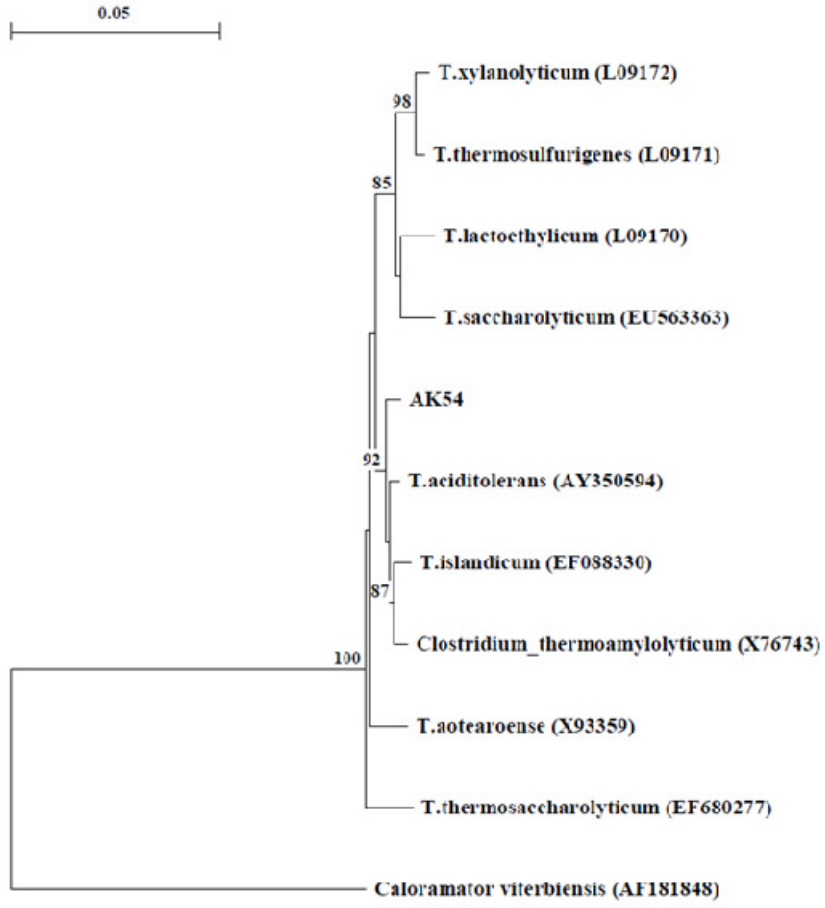

Figure 8. Phylogeny of the strains based on the 16S rRNA gene sequences (1400 bp). The phylogenetic tree was generated using a distance matrix and neighbor joining algorithms with 500 bootstraps. Only supported bootstrap values (>80%) are shown. *Caloramator viterbenses* (AF181848) was selected as an out-group. The scale bar indicates 0.05 substitutions per nucleotide position.

Temperature and pH ranges. The strain had a relatively narrow temperature growth range or 55.0°C to 70.0°C with optimal temperature being 65.0°C (maximum growth rate; 0.642 h^{-1}). No growth was observed below 55.0°C or above 70.0°C (Fig. 2a). The pH optimum was 5.0. Below pH 4.0 and above pH 6.0 no growth was observed (Fig. 2b). The maximum growth rate (μ_{max}) at pH 5.0 was 0.614 h^{-1}. At pH 6.0 the growth rate was slightly lower than at pH 5.0 (0.474 h^{-1}).

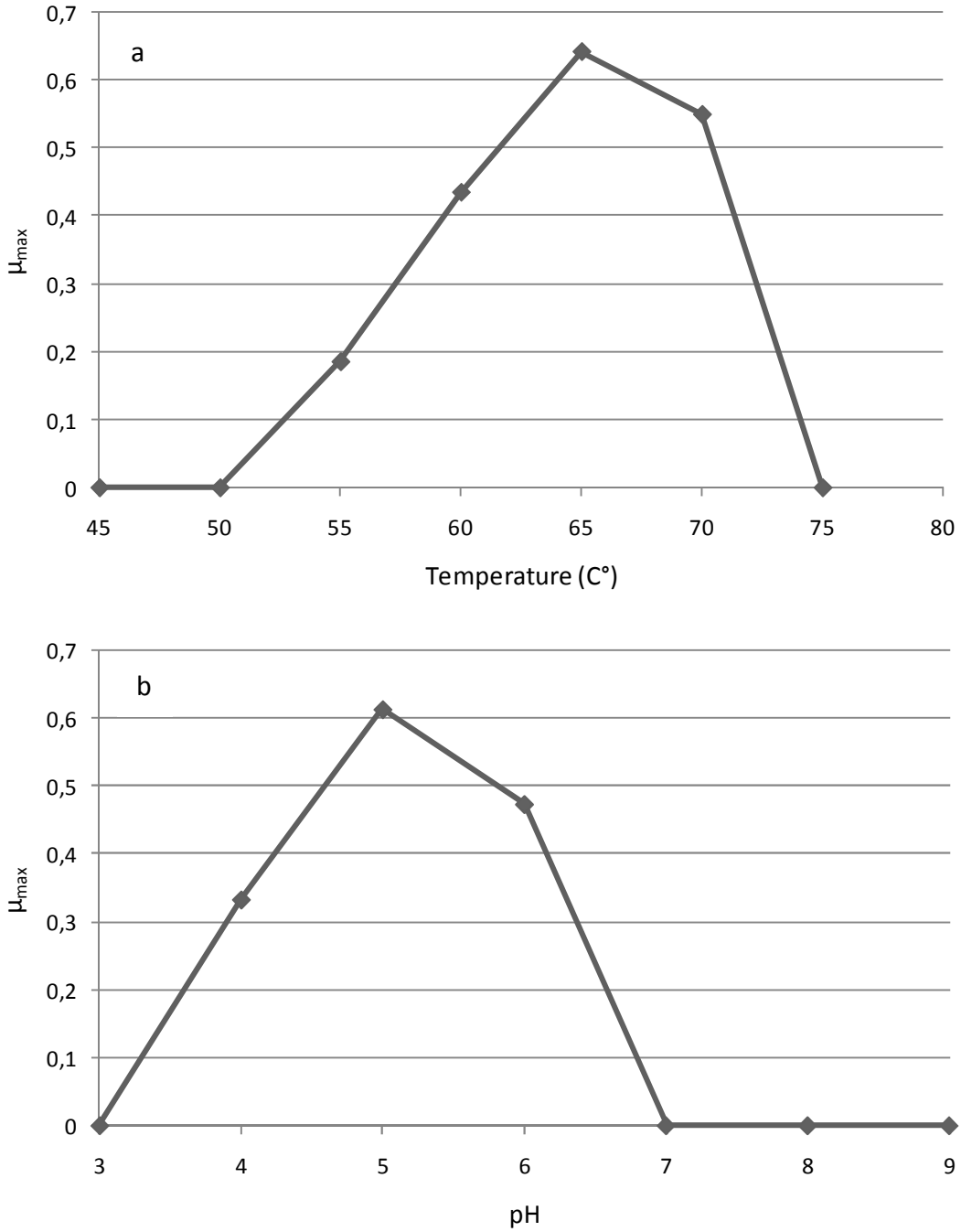

Figure 2. Optimum growth conditions for strain AK_{54}, temperature (a) and pH (b).

Effect of substrate concentration. Different initial glucose concentrations were used to investigate its effect on growth and end product formation for the strain.

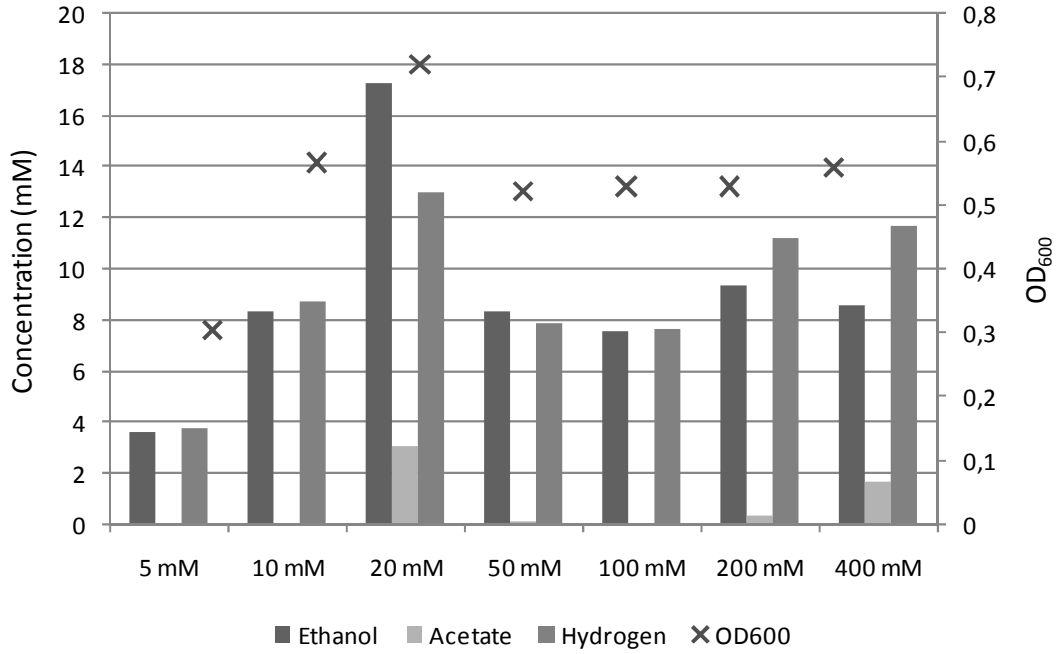

Figure 3. End product formation and growth from different substrate concentration for strain AK_{54}

A good correlation is observed between the initial glucose concentrations from 5 to 20 mM and the end product formation. The strain is producing 0.8 to 0.9 mol-EtOH and 0.7 to 0.8 mol-hydrogen mol glucose^{-1}. Acetate was only a minor product for the strain. However, at glucose concentration above 20 mM, a clear inhibition is observed. This can be seen by lower amounts of end product formation (Fig. 3) as well as insufficient glucose degradation (Table 1).

Table 5. Glucose concentration and carbon recovery. N.c. = not calculated.

Initial glucose concentration (mM)	Glucose concentration after fermentation (mM)	Carbon recovery (%)
5	0.4	39.7
10	0.2	42.6
20	0.6	52.6
50	31.5	23.1
100	99.3	N.c.
200	199.3	N.c.
400	398.3	N.c.

Highest hydrogen production was achieved when the glucose concentration was 20 mM (13.0 mmol H_2 L^{-1}). Ethanol production decreases from 17.3 mM to 8.3 mM when glucose concentration is increased from 20 to 50 mM, respectively. This is also shown by less biomass produced at high glucose loadings. When glucose concentration was increased to 200 and 400 mM the hydrogen production increased slightly compared to 50 and 100 mM although being less than at 20 mM. At glucose concentration 5 to 20 mM the carbon recovery is surprisingly low although the glucose is almost completely degraded. Other end products (lactate, formate or butyrate) were not detected, however, possible production of pyruvate and succinate was not measured and could thus explain this.

Substrate utilization. Of the carbon sources tested, strain AK_{54} only utilized the sugars (Fig. 4).

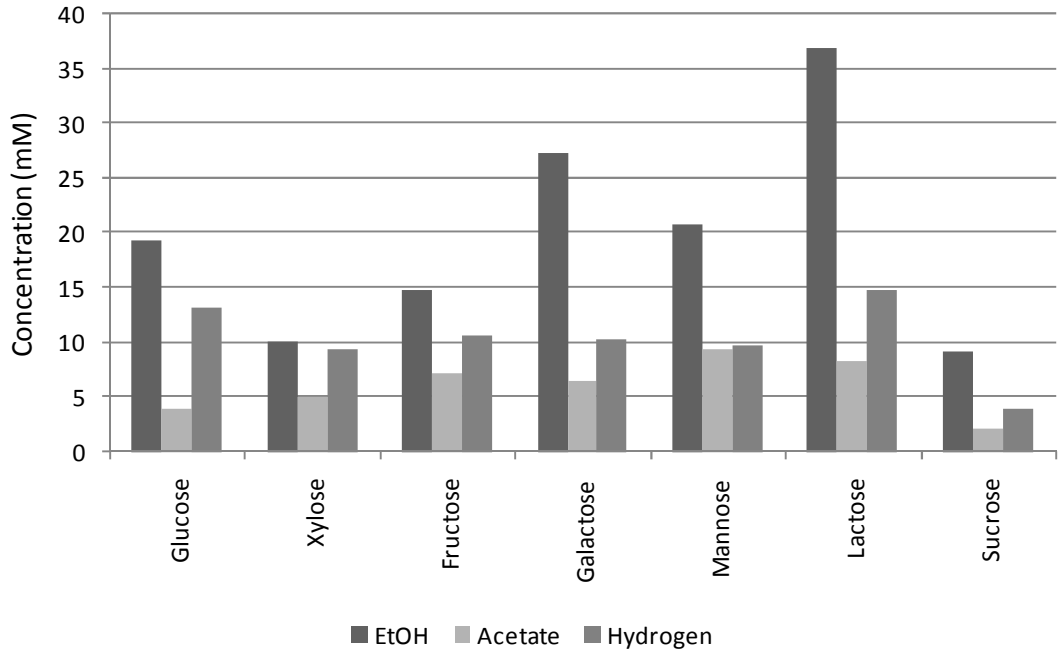

Figure 4. End product formation from different substrates for strain AK_{54}.

Fermentation of 20 mM of various C_5, C_6 and C_{12} sugars showed that most ethanol and hydrogen were produced from the disccharide lactose or 37.0 mM and 14.9 mmol L^{-1}, respectively. On glucose, ethanol and hydrogen production were 19.3 and 13.2 mmol L^{-1}, respectively. These results show that the strain is saccharolytic and can use both mono- and disaccharide but none of the complex polymers tested. Carbon recovery and electron balance for growth on the saccharides tested were calculated from the data in Figure 4 (Table 2).

Table 6. Carbon and electron recovery for growth on saccharides for AK_{54}.

Substrate	Carbon recovery (%)	Electron recovery (%)
Glucose	58.1	66.9
Xylose	46.0	36.3
Fructose	66.6	53.0
Galactose	84.5	79.9
Mannose	75.9	69.5
Lactose	56.7	54.0
Sucrose	14.3	13.7

Relatively low carbon recovery was in general obtained on all sugars, the highest recovery was observed from galactose where the electron recovery was also the highest. The lowest carbon recovery was obtained from sucrose which is not surprising since end product formation from sucrose is very low. The electron recovery was in general relatively low. This is surprising and not easy to explain since no other reduced end products were detected in present study. One explanation might be that the substrates were not completely degraded but only glucose was measured of the substrates in Table 2.

Kinetics of glucose degradation to various end products was investigated (Fig. 5). Glucose was completely degraded in 24 hours. The doubling time was 1.01 h (μ_{max} = 0.684 h^{-1}). The main end products produced were ethanol (21.3 mM) and hydrogen (15.6 mmol L^{-1}). The ethanol production rate was 2.5 mM EtOH h^{-1} and hydrogen 2.1 mmol H_2 $L^{-1}h^{-1}$.

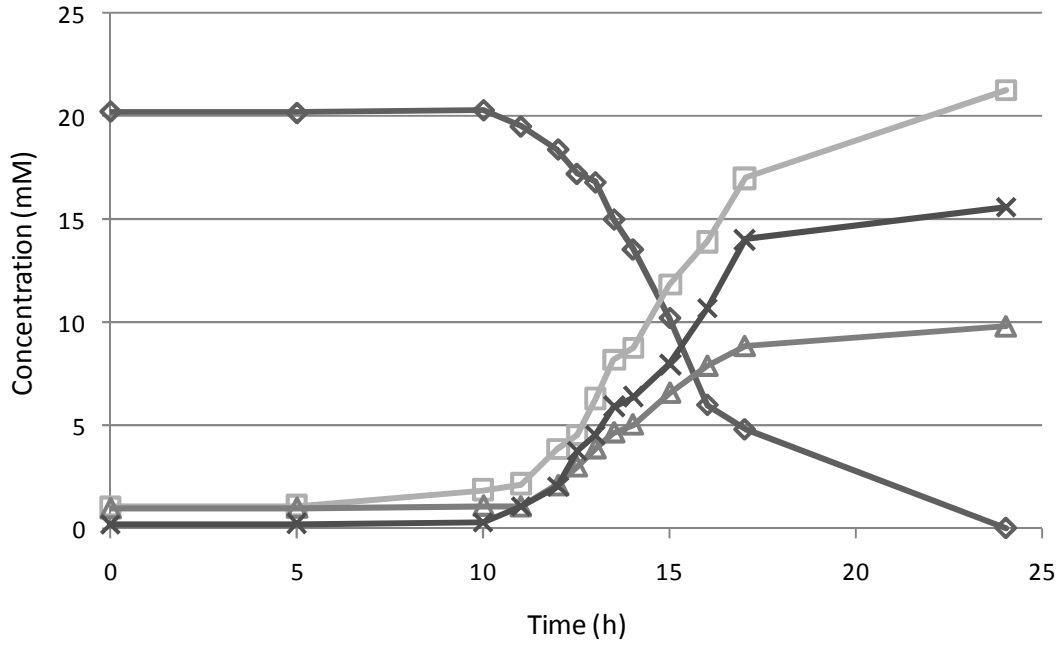

Figure 5. Growth of strain AK_{54} with glucose as carbon source;
Δ= acetate, □ = ethanol, x = hydrogen, ◊ = glucose. Control sample was subtracted.

Effect of partial pressure of hydrogen on hydrogen production. To investigate the influence of the partial pressure of hydrogen (pH_2) on hydrogen production, the strain was cultivated using different liquid/gas volume ratios (Fig. 6).

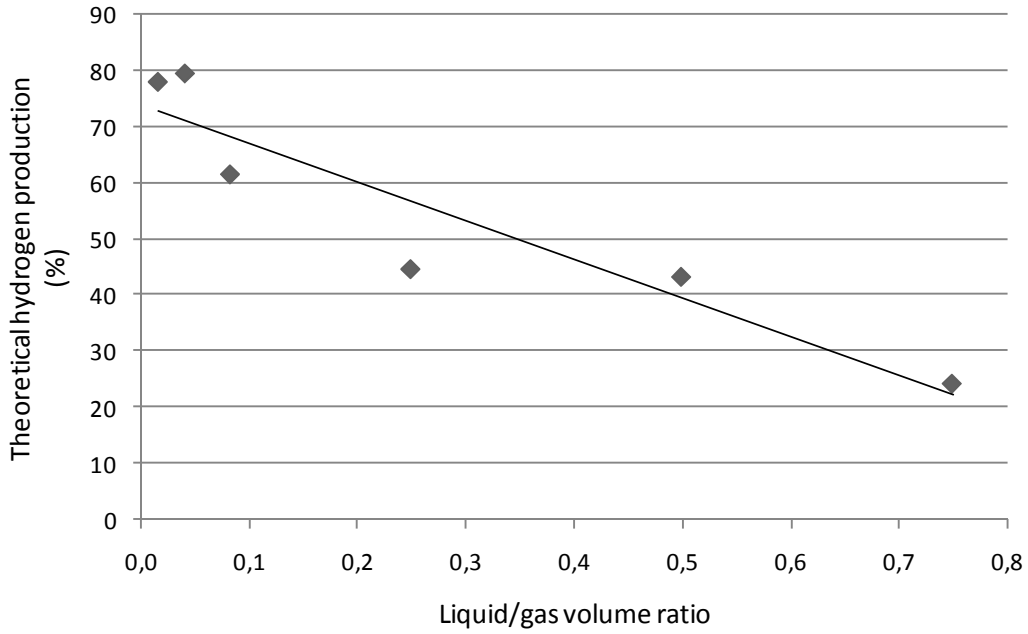

Figure 6. Effect of liquid/gas volume ratio on hydrogen production for strain AK_{54}.

The strain produces 80.0% of the theoretical yield of hydrogen with the lowest liquid/gas phase ratio, dropping to only 24.1% with the highest ratio. The liquid/gas volume ratio clearly affects the production of other end products (Fig. 7).

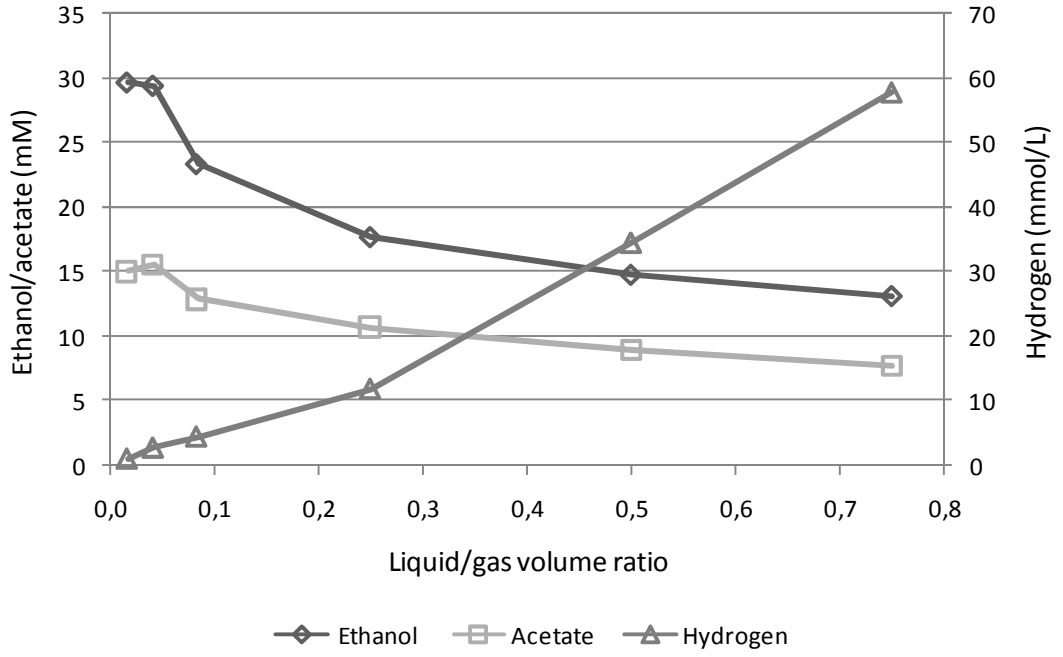

Figure 7. Effect of liquid/gas volume ratio on end product formation for strain AK_{54}.

Ethanol and acetate production decrease when the liquid/gas ratio is increased. Hydrogen concentration increases from 1.1 mmol H_2 L^{-1}, where the liquid/gas phase ratio is to lowest, to 57.9 mmol H_2 L^{-1} where the ratio is the highest. Glucose was completely degraded in all cases. Production of other end products were not measured but lactate is a possible product produced at higher liquid/gas ratio.

End product formation from hydrolysates

Strain AK_{54} was inoculated in BM medium containing 30% hydrolysates (7.5 g L^{-1}) from different types of complex biomass (cellulose, hemp leafs, hemp stem fibers, NPi, BS and grass). Highest ethanol production was observed on cellulose and grass where H_2SO_4 was used as pretreatment (Table 3). The amount of end products produced from cellulose is in good correlation to what was observed from glucose alone (Fig. 5). Ethanol production from hemp leafs was much lower than from hemp stem fibers when heat was the only pretreatment. The use of H_2SO_4 increased the ethanol production from hemp leafs from 3.00 mM to 10.9 mM and also had a great influence on straw where the ethanol production increased from 5.4 mM to 17.9 mM. Hydrogen production never yielded more than 5 mmol L^{-1}.

Table 7. End product formation from 30% hydrolysates.

	End products (mM)				
Biomass	**Ethanol**	**Acetate**	**Hydrogen**	**Initial glucose concentration (mM)**	**Glucose concentration after fermenation (mM)**
Cellulose	24.2	13.0	5.1	35.0	18.6
Cellulose + H_2SO_4	25.3	13.3	5.3	29.5	6.9
Cellulose + NaOH	24.3	14.0	3.9	40.0	18.9
BS	5.4	4.3	1.5	7.8	0.1
BS + H_2SO_4	17.9	14.1	3.2	17.7	0.0
BS + NaOH	19.4	17.1	4.5	22.8	0.2
Hemp leaf	3.00	4.2	0.7	7.0	0.2
Hemp leaf + H_2SO_4	10.9	9.1	2.5	15.4	0.0
Hemp leaf + NaOH	0	2.0	0	1.9	0.1
Hemp stem fibers	14.5	10.9	2.9	18.2	0.0
Hemp stem fibers + H_2SO_4	18.0	13.8	3.3	19.7	0.0
Hemp stem fibers + NaOH	20.5	18.3	3.7	25.8	0.0
NPi	9.6	6.9	2.3	9.5	0.8
NPi + H_2SO_4	10.1	8.6	2.6	16.6	0.0
NPi + NaOH	8.5	7.7	1.6	10.9	0.0
Grass	15.1	12.3	0	13.5	0.0
Grass + H_2SO_4	24.9	15.8	3.5	24.4	0.0
Grass + NaOH	22.3	17.9	3.8	22.8	0.0

Discussion

Hydrogen and ethanol producing bacteria have gained much attention in recent years because of increased interest in renewable energy sources. High values of hydrogen produced per mol of glucose utilized have been reported from the hyperthermophiles *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii*; 3.3 mol-H_2 mol-carbohydrate^{-1} (van Niel *et al.*, 2002). High yields of hydrogen have also been observed from enrichment culture from Icelandic hot spring, 3.2 mol-H_2 mol-glucose^{-1} in semi continuous batch reactor and 2.10 mol-H_2 mol-glucose^{-1} in batch culture (Koskinen *et al.*, 2008a; Koskinen *et al.*, 2008b). The highest ethanol yield reported is for the thermophilic bacteria *Thermoanaerobacter ethanolicus*, 1.9 mol-EtOH mol-glucose^{-1} degraded (Wiegel & Ljungdahl, 1981)

Strain AK_{54} was isolated from Icelandic hot spring with glucose as carbon source. Hydrogen production capability of the isolate on mono- and disaccharides and hydrolysates from various complex biomasses was studied in batch cultures. The temperature in the hot spring from where the strain was isolated was 66.0°C and the pH 5.27. When isolated, the temperature was slightly below the natural environmental temperature, 60°C, and pH slightly higher, pH 6.0. Thus, it was not surprising that the strain had similar temperature and pH optimum as the natural environmental conditions (Fig. 2).

High initial substrate concentration can play an important role in ethanol and hydrogen production (Kumar & Das, 2001; Lacis & Lawford, 1988; van Ginkel & Sung, 2001; Sommer *et al.*, 2004). However, fermenting microorganisms can have limited tolerance towards increased substrate loadings (Olsson & Hahn-Hägerdal, 1996). This was clearly observed in present study when strain AK_{54} was cultivated on different initial concentrations of glucose varying from 5 – 400 mM. Results show that ethanol and hydrogen production increases when glucose concentration is 5 – 20 mM (Fig. 3). Above 20 mM a inhibition is observed which could possibly be explained by substrate inhibition (van Ginkel & Logan, 2005).

It has been reported that partial pressure of hydrogen affects the growth and production of hydrogen (Nath & Das, 2004; Hawkes e*t al.*, 2002). This was observed for strain AK_{54} in present study where the theoretical yield of hydrogen increased from 24.1% to 80.0% when the liquid/gas volume ratio was changed from 0.75 to 0.02 (Fig. 6).

Figure 4 shows end product formation for strain AK_{54} from mono- and disaccharides tested. The strain produced 0.51 mol-H_2 for every mol of glucose degraded, which is only 12.7% of theoretical yield. In another experiment where glucose degradation was followed on time (Fig. 5), the hydrogen yield was slightly higher or 0.78 mol-H_2 mol-glucose^{-1} (19.5% of theoretical yield). Both these experiments were carried out in serum bottles with liquid/gas volume ratio of 0.5 and by lowering the partial pressure of hydrogen the yield could was increased to 80% (Fig. 6).
Ethanol production from glucose was 1.1 mol-EtOH mol-glucose^{-1} or 55.5% of theoretical yield (Fig. 4). Thus, strain AK_{54}, is a good ethanol producer as well and could even be better suited for ethanol than hydrogen production.

When lignocellulosic biomass is hydrolysed the main sugars are glucose and xylose (Hamelinck *et al.*, 2005), both of which strain AK_{54} degrades. When choosing the amount of hydrolysate added to the media, 7.5 g L^{-1} was chosen since substrate concentration in more amount than 20 mM (as glucose) has inhibitory effect on the strain (Fig. 3). The Whatman paper consists of 99% glucose; thus, the glucose concentration in 7.5 g L^{-1} HL should be 41.7 mM if all the glucose bound in the cellulose paper was released during the hydrolysis. Strain AK_{54} produced 21.3 mM ethanol from 20 mM glucose (Fig. 5), giving the stochiometry:

1.0 Glucose → 1.1 EtOH + 0.49 Acetate + 0.78 H_2 + 1.56 CO_2

From the cellulose HL the strain produced 24.2 – 25.3 mM ethanol, depending on the pretreatment used. According to the equation above, 41.7 mM glucose should result in 45.9 mM of ethanol. Clearly the strain does not degrade all the glucose in the hydrolysate. This can be seen by partial glucose degradation as well as ethanol

production which was approximately 24 – 25 mM (Table 3). The final pH in the hydrolysates was not measured but could have affected the ethanol production. In the other HL's the glucose was completely degraded, however, less ethanol was produced than expected from the strain's theoretical capacity.

It has been reported that pretreatment is important when wheat straw is used in ethanol production (Saha *et al.*, 2005). This was clearly observed in present study on BS where the ethanol production increased from 5.4 mM (only heat pretreatment) to 17.9 – 19.4 mM where H_2SO_4 and NaOH were used, respectively. The pretreatment also affected ethanol production from grass where it increased from 15.1 mM to 24.9 mM when H_2SO_4 was used. In other HL's, the pretreatment had less effect, especially on NPi where ethanol production was similar in all cases, varying from 8.5 – 10.1 mM (Table 3).

Phylogenetic studies on strain AK_{54} reveales that the bacterium belongs to the genus *Thermoanaerobacterium*. The closest phylogenetic relative was *Thermoanaerobacterium acidotolerance* (99% homology), a well known ethanol and hydrogen producer (Kublanov *et al.*, 2007). Other strains closely related to strain AK_{54} (0.5 – 1.7% difference within the strains) are *Clostridium thermoamylolyticum*, *T. aotearoense*, *T. islandicum* and *T. thermosulfurigenes*. All have similar phenotypic characteristics as strain AK_{54} and most of them have been reported to produce ethanol and hydrogen (Liu *et al.*, 1996; Orlygsson & Baldursson, 2007; Lee *et al.*, 1993).

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Thermoanaerobacterium thermosulfurigenes comb. nov., and *Thermoanaerobacter thermohydrosulfuricus* comb. nov., respectively; and transfer of *Clostridium thermohydrosulfuricum* 39E to *Thermoanaerobacter ethanolicus*. International Journal of Systematic Bacteriology, *43*, 41-51.

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7. Conclusion

The original objective was to isolate thermophilic bacteria that were able to degrade glycerol and produce 1,3-propanediol. Although this objective was not reached it yielded two new species of bacteria that are described in this thesis.

In present study, three thermophilic, anaerobic strains were isolated. Phylogenetic analysis on pure cultures revealed two new species within the genus *Caloramator*. Strains AK_{44} and AK_{35} have 94.5% and 92.1% homology to *C. viterbensis*, respectively. The homology between the two strains is 96.5% (appendix I). These two strains are presented in chapter 5 as a manuscript of scientific paper. The other strain, AK_{54}, falls within the genus *Thermoanaerobacterium* and was investigated in details with respect to hydrogen and ethanol production. Strain AK_{54} has 99% homology to *T. aciditolerans* (appendix II).

Physiological studies were described for all strains concerning optimal growth conditions, effect of increased substrate concentration, ethanol tolerance, substrate utilization and kinetics of glucose degradation and end product formation. In addition strain AK_{54} was investigated further with respect to hydrogen production; the effect of partial pressure of hydrogen on hydrogen production was tested and ethanol and hydrogen production from hydrolysates made from various complex biomasses was investigated.

Strains AK_{44} and AK_{35} have similar characteristics compared to their closest relative concerning optimal growth conditions and generation times. However, there is difference in their substrate utilization. Strain AK_{44} was isolated on glycerol, thus it was not surprising that growth was observed when glycerol was used as a substrate. The main end product produced was ethanol but 1.3-propanediol was not detected. The strain was saccharolytic and able to degrade several mono- and disaccharides to ethanol and small amount of acetate and hydrogen. Strain AK_{35} did not grow on glycerol but degraded mono- and disaccharides; the main end products were acetate and hydrogen. Strain AK_{44} was a good ethanol producer, producing 80% of theoretical

yield. It would be interesting to investigate its ability to degrade complex biomass to see if the strain is well suited for ethanol production from lignocellulosic biomass. Increased substrate concentration clearly affected the ethanol production, thus, it would be interesting to change the culture conditions to fed-batch or continuous cultures in order to enhance the substrate concentration.

Strain AK_{54} produced ethanol and hydrogen from all the hydrolysates tested except for hemp leafs and barley straw where no chemical pretreatment was used. The pretreatment clearly played an important role, especially for grass and barley straw where the ethanol production was almost doubled. Different liquid/gas ratio affected the hydrogen capacity for strain AK_{54}. Where the ratio was the lowest, the strain produced 80% of theoretical yield of hydrogen, dropping to approximately 25% with the highest ratio. The strain did not have a broad substrate range and of the substrates tested it was only capable of degrading the sugars. Furthermore, the carbon recovery and electron balance was relatively low which is not easy to explain since no other end products were detected, e.g. lactate, formate or butyrate. It would also be interesting to change the culture conditions for this strain to fed-batch or continuous in order to enhance its substrate tolerance.

8. Appendixes

Appendix I

Seq->	AK_{44}	AK_{35}
AK44	ID	0.965
Caloramator_viterbiensis (AF181848)	0.945	0.921
Caloramator_uzoniensis (AF489534)	0.886	0.888
Caloramator_indicus (X75788)	0.886	0.887
Caloramator_coolhaasii (AF104215)	0.886	0.889
Thermobrachium_celere (DQ207958)	0.893	0.911
Clostridium_fervidus (L09187)	0.869	0.877
Oxobacter_pfennigii (X77838)	0.831	0.835
Clostridium_butyricum (EU183474)	0.832	0.824
Moorella_thermoacetica (AY656675)	0.806	0.803
Moorella_thermoautotrophica (X77849)	0.794	0.792
Moorella_glycerini (U82327)	0.804	0.8
Caloramator_proteolyticus (X90488)	0.874	0.875
Clostridium_acetobutylicum (X78070)	0.83	0.827
AK35	0.965	ID
Escherichia coli (EU014689)	0.735	0.742
Caloramator australicus (EU409943)	0.871	0.88

Appendix II

Seq->	**AK_{54}**
AK54	ID
Clostridium_thermoamylolyticum (X76743)	0.985
T.aciditolerans (AY350594)	0.99
T.aotearoense (X93359)	0.981
T.islandicum (EF088330)	0.987
T.saccharolyticum (EU563363)	0.974
T.thermosulfurigenes (L09171)	0.975
T.lactoethylicum (L09170)	0.972
T.xylanolyticum (L09172)	0.972
T.thermosaccharolyticum (EF680277)	0.973
Caloramator viterbiensis (AF181848)	0.839