University of Akureyri
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BioEthanol:

Fuel of the future ?

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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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Hilma Eiðsdóttir Bakken

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

Microbial fermentations are potential producers of sustainable energy carriers. In this study, 68 samples were used for the isolation of ethanol and hydrogen producing bacteria from various carbon substrates from geothermal springs in Iceland. 16S rRNA analysis revealed that most of low temperature (50°C) enrichments indicated the presence of bacteria belonging to *Thermoanaerobacterium*, *Caloramator* and *Clostridium*. At higher temperatures (60°C) *Thermoanaerobacterium* and *Thermoanaerobacter* became more dominant and at 70 and 75°C only members of the genera *Thermoanaerobacter* and *Caldicellulosiruptor* were present. Low temperature enrichments (50°C) produced mainly ethanol, acetate and butyrate from monosugar fermentation and two enrichments produced more than 1.3 mol-EtOH/mol-glucose and two enrichments produced more than 1.0 mol-EtOH/mol xylose. Most of the enrichments that directed their carbon flow to ethanol belong to *Caloramator*, *Paenibacillus* and *Thermoanaerobacterium*. At higher temperatures, several enrichments produced more than 1.0 mol-EtOH/mol-glucose and xylose, especially at 60°C, mostly belonging to *Thermoanaerobacter* and *Thermoanaerobacterium*. Butyrate was only detected in one of the 18 enrichments at 60°C and not at higher temperatures. Only one enrichment of a total of 16 at 70 and 75°C produced more than 1.0 mol-EtOH/mol glucose but the carbon flow was directed more towards acetate and hydrogen formation. Seven cultures were investigated further with respect to phylogeny and physiology. Six of the strains belonged to *Thermoanaerobacterium* and one to *Paenibacillus*. Basic characteristics concerning T_{opt} and pH_{opt} were performed on all seven strains, indicating that the *Thermoanaerobacterium* strain grow best at around 60°C and at low pH between 5 and 6. The *Paenibacillus* strain was a moderate thermophile (T_{opt}; 50°C) and had a pH_{opt} at 8.0. *Paenibacillus* was the fastest growing bacterium with the shortest generation time of 0.39 h (pH_{opt}) and 0.71 h (T_{opt}). The *Thermoanaerobacterium* strains showed generation times between 0.81h to 1.99h. All strains were highly saccharolytic degrading broad spectrum of pentoses, hexoses and disaccharides as well as xylan and pectin. All strains produced more than 1 mol-EtOH/mol-glucose and xylose and could grow in the presence of 3.2% ethanol (v/v) but not at 6.4%. Different initial substrate loadings (glucose) indicated that at glucose concentrations between 50 and 100 mM, a clear inhibition occurred leading to inefficient glucose degradation and lower end product formation. Growth of two strains

AK_{66} and AK_{17} was also investigated on hydrolysates (0.75% w/v) made from cellulose (Whatman paper), newspaper, hemp leaves and hemp fibers (*Cannabis sativa*), barley straw (*Hordeum vulgare*) and grass (*Phleum pratense*). End product formation was analyzed from the cultures after fermentation. Strain AK_{66} produced most ethanol (20 to 24 mM) from cellulose but weak acid and base pretreatment did not enhance ethanol yields. On other biomass types, lower ethanol yield were observed (lowest on barley straw; 4 mM, highest on hemp stem, 12 mM) but were increased significically by pretreatments. Yields of ethanol by strain AK_{17} were considerable higher, ranging from 5 mM on hemp leaves to 50 mM on pure cellulose. Acidic pretreatment enhanced ethanol yields mostly on hemp leaves (5 mM to 18 mM) and base pretreatment on grass (20 mM to 28 mM). Other products from hydrolysate fermentation for both strains were acetate, carbon dioxide and hydrogen, but in lower amounts.

Key words: hot spring, thermophilic, complex biomass, ethanol, 16S rRNA

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I would like to thank Dr. Jóhann Örlygsson for the opportunity to work on this project, his support and guidance, without which I would not receive this degree. I would also like to thank my coworkers, friends and helpers at the lab: Steinar Rafn Beck, Máney Sveinsdóttir, Margrét Auður Sigurbjörnsdóttir and Hildur Vésteinsdóttir. The staff of Prokaria also gave me invaluable training and advice.
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Thank you all,
Hilma E. Bakken
May 15th 2009

Útdráttur

Gerjun örvera er ein leið til þess að framleiða endurnýjalega orkugjafa. Í þessari rannsókn voru 68 sýni úr Íslenskum hverum notuð til að einangra hitakærar bakteríur sem framleiða vetni og etanól. 16S rRNA hlutraðgreining leiddi í ljós að auðgunarsýni úr lágum hita (50°C) innihéldu bakteríur sem tilheyra ættkvíslum *Thermoanaerobacterium*, *Caloramator* og *Clostridium*. Við hærri hita (60°C) verða *Thermoanaerobacterium* og *Thermoanaerobacter* meira áberandi og við 70 til 75°C eru eingöngu bakteríur innan ættkvísla *Thermoanaerobacter* og *Caldicellulosiruptor* til staðar. Við lágt hitastig (50°C) framleiddu ræktirnar aðallega etanól, ediksýru og smjörsýru (auk H_2 og CO_2) og tvær ræktir framleiddu meira en 1,3 mól-EtOH/mól-glúkósa og tvær ræktir framleiddu meira 1,0 mól-EtOH/mól xýlósa. Flestar ræktirnar sem beindu kolefnaflæði aðallega í etanól tilheyra ættkvíslum *Caloramator*, *Paenibacillus* og *Thermoanaerobacterium*. Við hærri hitastig framleiddu nokkrar ræktir (aðallega *Thermoanaerobacter* og *Thermoanaerobacterium*) meira en 1,0 mól-EtOH/mól-glúkósa og xýlósa, sérstaklega við 60°C. Smjörsýra var aðeins til staðar í einu sýni af 18 við þetta hitastig. Einungis ein rækt af sextán við 70 til 75°C framleiddi meira en 1,0 mól-EtOH/mól en kolefnaflæðið var meira í áttina að ediksýru og vetni. Sjö ræktir voru rannsakaðar nánar m.t.t. flokkunar- og lífeðlisfræði þeirra. Sex stofnanna tilheyrðu ættkvíslinni *Thermoanaerobacterium* og ein *Paenibacillus*. Rannsóknir á T_{opt} og pH_{opt} leiddu í ljós að *Thermoanaerobacterium* stofnarnir uxu best við 60°C og við pH á milli 5 og 6. *Paenibacillus* stofninn óx best við 50°C og við pH 8,0 en þessi stofn óx hraðast allra sjö stofnanna (kynslóðatími á milli 0,39 og 0,71 klst). Thermoanaerobacterium stofnarnir voru með kynslóðatíma frá 0,81 til 1,99 klst. Allir stofnarnir uxu vel á flestum pentósum, hexósum og tvísykrum auk xýlans og pektíns og framleiddu meira en 1 mól-EtOH/mól-glúkósa og xýlósa auk þess að geta vaxið í viðurvist 3,2% etanóls (v/v) en ekki í 6,4% styrk. Mismunandi upphafsstyrkur glúkósa leiddi í ljós að við styrk á bilinu 50 til 100 mM, varð greinileg hindrun, þ.e. glúkósi var ekki fullkomlega brotinn niður og mun minna var framleitt af lokaafurðum. Vöxtur tveggja stofna AK_{66} and AK_{17} var einnig rannsakaður á „hýdrólýsötum“ (0,75% w/v) útbúnum úr sellulósa (Whatman paper), dagblaðapappír, hampi (lauf og stönglar) (*Cannabis sativa*), bygghálm (*Hordeum vulgare*) og grasi (*Phleum pratense*). Framleiðsla lokaafurða var könnuð eftir gerjun. Stofn AK_{66} framleiddi mest etanól (20

til 24 mM) úr cellulósa en væg sýru og basaformeðhöndlun jók ekki etanólframleiðsluna. Á öðrum gerðum lífmassa fengust lægri gildi (minnst úr bygghálmi; 4 mM, mest á hampstönglum (12 mM) en jókst verulega við formeðhöndlun. Etanólframleiðsla stofns AK_{17} var mun hærri eða frá 5 mM á hampi (lauf) til 50 mM á hreinum cellulósa. Sýruformeðhöndlun jók etanóframleiðsluna aðallea á hampi (lauf) eða úr 5 í 18 mM en basaformeðhöndlun jók etanólframleiðsluna mest á grasi eða úr 20 í 28 mM. Aðrar afurðir sem voru mældar eftir gerjun beggja stofnanna á hýdrólýsötum voru ediksýra, vetni og koltvísýringur en í mun minna mæli.

Lykilorð: hverir, hitakær, flókinn lífmassi, etanól, 16S rRNA

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1. Background and research objectives

The present studies are rooted in earlier projects called “Energy from paper” that started in 2006, which led to the present “BioEthanol” project which started in 2007, both performed at The University of Akureyri. The objective of these projects were to isolate and characterize ethanol-producing thermophiles on various carbohydrates. Strains were isolated on several types of sugars and polymers, i.e. glucose, xylose, pectin, xylan and cellulose (paper and powder). The ethanol production of all enrichment cultures were investigated as well as their phylogenetic relationship. The results are presented as a manuscript of a scientific paper (Chapter 5). Based on phylogeny and ethanol production, one strain was selected and compared to strain Ak_{17}, an isolate already at our laboratory, on hydrolyzates from complex biomass. The results of which are presented as a manuscript of a scientific paper (Chapter 6).

2. Introduction

Today most energy demands are met by non-renewable energy sources that may well lead to resource depletion and environmental problems. Global warming is evident and there is high confidence that human activities have contributed to it (IPCC, 2007). A demand to develop novel renewable energy harvesting technologies is urgent and the production of ethanol from biomass has been focused upon recently (Wyman, 2007) (Sánchez & Cardona, 2008). Most of the ethanol produced from biomass today is however produced from starch based plants, i.e. corn maiz and sugarbeet/sugarcane causing rise in food and feed prices. This first generation ethanol production has therefore caused a huge debate because of raised food prices and the consequences of the competition for land between food and fuel producers. Therefore, more focus has recently been on the production of second generation ethanol from lignocellulosic biomass such as wood and agricultural residues, energy crops and residues from the paper and pulp industry. This type of biomass is however more difficult use as substrate for ethanol production and requires substantial pre-treatment that may be very costly. Additionally, lignocellulosic material contains many carbohydrates that are not degraded by yeast traditionally used for the first generation ethanol production. Therefore, the focus upon thermophilic, fermentative bacteria has been increasing for the past few years. Many of these bacteria have active polymer degrading enzyme systems (cellulases, xylanases, pectinases) and could be potential producers for second generation of ethanol (Collins, Gerday, & Feller, 2004). Additionally, many of these bacteria can degrade pentoses (e.g. xylose and arabinose), that are part of the hemicellulosic part of lignocellulose.

2.1. Biofuels

Fuels made from recently dead biomass qualify as biofuel. Renewable refers to the “closed” short cycle of carbon (1-10 years) versus the “open” cycle of fossil fuels (millions of years). With closed cycle there is a balance between the carbon bound while the plant is growing and the carbon released when the fuel is utilized, and these actions are temperally not so far apart as with fossil fuels, which are generated at much slower rate than they are utilized. Biofuels are both gaseous (biomethane, biohydrogen) and liquid (biodiesel, biomethanol, biobutanol and bioethanol). Present investigation focuses upon bioethanol. Biofuels do have the disadvantage to fossil fuels that they are

produced from biomass that needs large area for cultivation on the surface of the earth, as opposed to fossil fuel which are buried deep under the surface. This is the biggest problem regarding biofuels, how to maximize the yield from our land area and balance the competition for land for food and fuels. The yield can be calculated in many ways and is affected by many factors, such as growth rate of the biomass, pretreatments applied and conversion rate of the fermenting organisms and finally the distillation process.

2.1.1. Bioethanol

Ethanol (C_2H_4OH) is a colorless, flammable and volatile liquid with boiling point at 78.4°C and a melting point at -112.3°C (Kosaric & Vardar-Sukan, 2001). Bioethanol is ethanol produced from biomass. It is usually produced by fermenting micro-organisms. Today the world ethanol production is about 51 billion liters and about 98% is produced by yeast from starch and sugar based livestock. Fuel ethanol is about 73% of the production, 17% is beverage and 10% industrial ethanol. The largest producers of bioethanol are USA and Brazil using corn and sugarcane, respectively (Renewable Fuels Association, 2007).

Ethanol as biofuel can be utilized either directly as fuel or as a gasoline enhancer. As biofuel, ethanol can be used on its own (100% ethanol) or in various blends (usually with gasoline). The most common blends are E10, E20, E30, E40 and E85. The E10 blend consists of 10% ethanol and 90% unleaded gasoline, whereas E85 consists of 85% ethanol and 15% gasoline. In America about 70% of the gasoline contains ethanol, mostly as E10, which vehicles can utilize without modifications to the engine. Higher concentrations of ethanol require modified vehicle engines (American Coalition for Ethanol, 2009). When used as an oxygenate, ethanol is substituting MTBE (methyl tert-butyl ether). Ethanol has several advantages over MTBE, it has higher oxygen content which means that less amount of it is required to achieve the same effect. Higher oxygen percentage of ethanol gives better oxidation which results in less emission of CO and aromatics. Ethanol is a greater octane booster than MTBE and is not toxic and does therefore not contaminate water resevoirs in accidents where it leaks out into the nature. But then again ethanol is more expensive to produce than MTBE and when it is mixed with gasoline it conducts electricity, which MTBE does not. When ethanol is used instead of MTBE Reid vapor pressure is higher which results in greater

volatilization of the fuel and makes ozone and smog emission (Thomas & Kwong, 2001).

2.2. Biomass

Biomass has been defined as any plant derived material that can be used for energy (EU, 2003), and as such it is the fourth biggest energy source on our planet. Renewable energy has a carbon cycle of 1 – 10 years as opposed to millions of years for fossil fuels. The energy stored in biomass comes from the sun light by photosynthesis but the fertilizers used are mostly made from fossil fuels and that has to be considered when switching to renewable energy on large scale. Yearly biomass energy has been estimated around 2900 EJ. The worlds energy use in 2004 was around 500 EJ which is only 17% of potential biomass energy. The easiest way to harvest energy from biomass is to burn it for heat production. This is done in many places but is more common in undeveloped countries. Other ways are combustion, (needs oxygen), pyrolysis, (anaerobic, i.e. gasification), and microbial degradation, i.e. fermentation. There are many types of biomass that can be used and different biofuel types that can be produced. Wet biomass includes animal manures, municipal solid wastes and green crops. These can be subject to anaerobic digestion for the production of methane and further heat, electricity, turbines, internal cumbustion engines, process streams, methanol and more. Forest and agricultural biomass include forest arisings, wood residues, cereal straw and woody biomass crops. These all contain lignocellulose which can be processed to solid fuel and methanol, and lignin to solid fuel and ethanol. Oil rich biomass comes from oil rich energy crops (vegetable oil) and meat processing (tallow, animal fat) which can be burned to generate heat or be subject to trans-esterification to produce fatty acid simple esters (biodiesel).

Different types of biomass are used in ethanol production i.e. starch and sugar based biomass, which require relatively little pretreatments and lignocellulosic biomass which is more complex and needs more extensive pretreatment. Examples of biomass used for ethanol production are e.g. maize, sugarcane, sorghum and mischantus.

2.2.1. Starch and sugar based biomass

Sugar (glucose) is the substance formed during the plants harvesting of the suns energy (photosynthesis) (Campbell & Reece, 2002):

$6\ CO_2 + 12\ H_2O + \text{Light energy} \rightarrow C_2H_{12}O_6 + 6\ O_2 + 6\ H_2O$

Plants store the energy in the form of sugars and starch. Starch is made up of chains of glucose. There are two chain types in starch, amylose, a linear polymer and amylopectin, a branced polymer. The sugar we know as "table sugar" is the disaccharide sucrose consisting of glucose and fructose. Sugar rich biomass is e.g. sugar cane and sugar beet (from both of which table sugar is made). Starch rich biomass types are i.e. potatoes, corn, wheat, cassava, rye, barley, triticale and sorghum. These biomass types mentioned above are used for first generation ethanol production. Second generation ethanol is however made from lignocellulosic biomass, which is the most abundant type of biomass on Earth (~50%).

2.2.1.1. Amylose and amylopectin

Amylose is the linear part of starch and is made up of glucose connected by α-1,4-glycosidic bonds. The amylopectin differs by the side chains, connected to "the-back-bone" by α-1,6-glycosidic bonds (fig. 2.1). Together these make up a tight matrix which is quite stable and specified enzymes are needed for its degradation (Grey, Zhao, & Emptage, 2006).

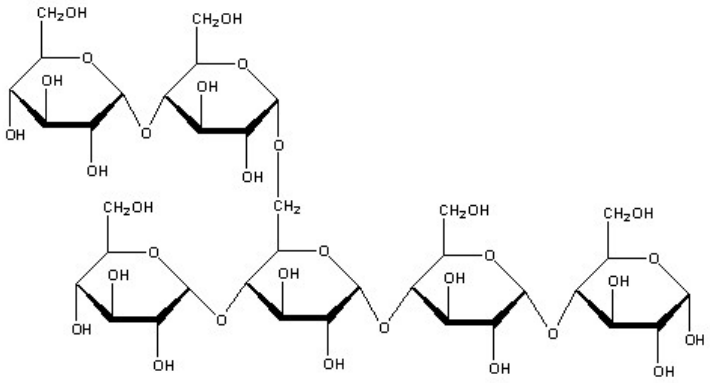

Figure 2.1. Amylopectin - the basic structure of starch

2.2.2. Lignocellulosic biomass

Lignocellulosic biomass is of various types, from grass and soft plants to hardwood and paper. Figure 2.2 shows the composition of a plant cell wall. Lignocellulose consists of cellulose, hemicellulose and lignin in various proportions depending on the type of biomass, it's age and various other conditions (Collins, Gerday, & Feller, 2004).

Lignocellulosic materials therefore need more extensive pretreatments to make the fermentable components accessible.

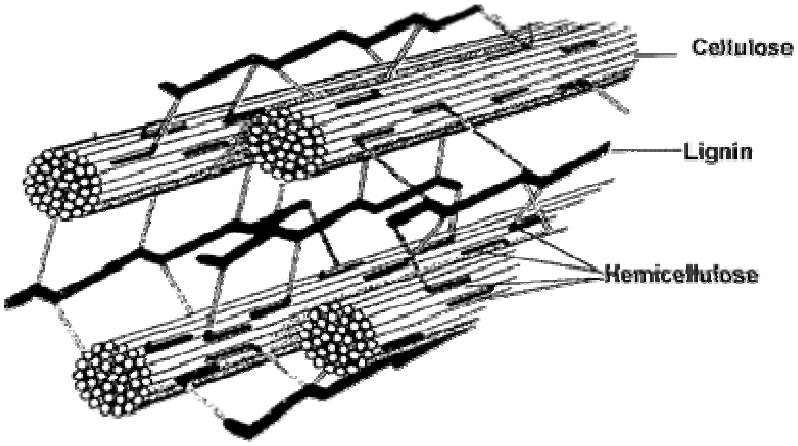

Figure 2.2. Composition of plant cell wall (Thostrup, 2006)(modified)

2.2.2.1. Cellulose

Cellulose is the main component of the cell wall in plants. It is a linear chain of glucose connected by β-1,4- glycosidic bonds, two glucose units together are called cellobiose and are the building block of cellulose (Fig. 2.3). Each glucose unit has a position of 180° compared to the next one. The polysaccharide is flat and can therefore be tightly packed. Each chain consists of 100 – 100,000 glucose units which bond within the chain by hydrogen bonds. Cellulose appears in two forms: amorphous and crystallic. Amorphous cellulose is irregular and easier to degrade than crystallic (Glazer & Nikaido, 2007).

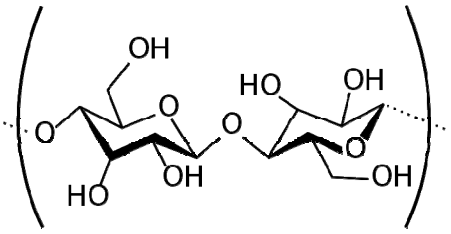

Figure 2.3. Cellobiose – the building block of cellulose (Johnson, 2001)

2.2.2.2. Hemicellulose

Hemicellulose is more complex than cellulose as it consists of many different types of sugars in branched chains. The main sugar is xylose, a five carbon sugar, but others e.g. are glucose, mannose, galactose, arabinose and rhamnose. Hemicellulose can be divided

into three groups, depending on the main sugar in the backbone, which is connected by β-1,4-glycosidic bonds. These groups are xylan, mannan and galactan (Brigham, Adney, & Himmel, 1996). Hemicellulose has different composition of these main groups, depending on the biomass. Softwoods (e.g. spruce and pine) have usually about 5-10% xylan, while hardwoods (e.g. aspen and oak) have 15-30% xylan (Filonova, et al., 2007).

2.2.2.3. Lignin

Lignin is the third component of lignocellulosic biomass, it's main function is to surround and protect the cellulose and hemicellulose from degradation of micro-organisms in plants. It constitutes around 20-30% of plant and wood biomass. Lignin is a complicated structure of various compounds (Fig. 4). The alcohols p-coumaryl-, coniferyl- and sinapyl- alcohol are the precursors of the most common building blocks in lignin. Softwood lignins are predominantly polymers of coniferyl alcohol. Hardwood lignins are made from coniferyl and sinapyl alcohol and lignin in grasses contain all three.

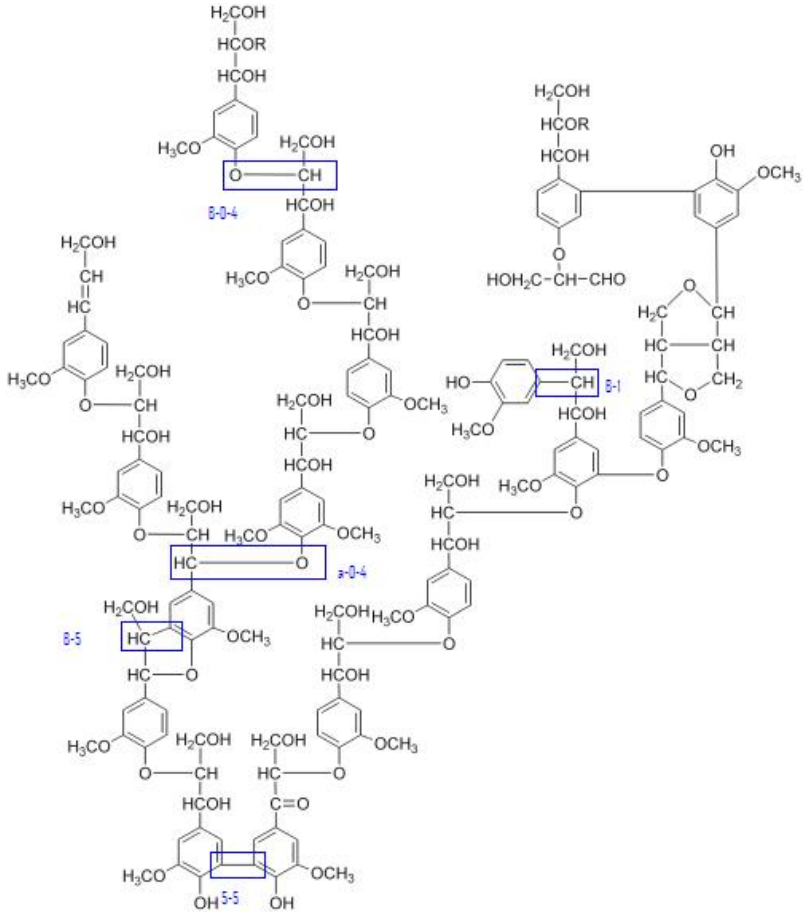

Figure 2.4. Lignin structure

The most abundant linkage (~50%) is the β-O-4 linkage. Others are β-5 (phenylcoumaran), 5-5'(biphenyl), α-O-4 (arylglycerol α-aryl ether) and β-1 (diarylpropane). Thus lignin has much more variety in types of linkages as compared to cellulose and hemicellulose and many enzymes are needed for its degradation. Lignin is very stable and difficult to degrade. No organisms is known to utilize lignin for growth, although white-rot fungi do degrade it (Fackler, et al., 2006). Lignin is formed by radical polymerization of the precursor alcohols and white-rot fungi degrade it by complex free radical chemistry and has most extensively be studied by the *Phanerochaete chrysosporium* (Glazer & Nikado, 1998) (Wariishi, et al., 1992) The problem of lignin degradation was dealt with by the paper industry long before ethanol production from biomass became important. Two major processes have been applied since 1874, firstly the "Sulfite Process", which uses calcium sulfite at acidic pH. This was largely overtaken about 1940 by the "Kraft Process", which uses alcalic conditions, heat and pressure, and does not degrade the cellulose as in the sulfite process (the yellowing of paper over time). In the Kraft Process the ether bonds are splitted by the strong nucleophilicity of the $^{-}$SH-ions (Kraft process, 2009) (Llamas, et al., 2007).

2.3. Pretreatment of biomass

Pretreatment of biomass are usually applied in order to reduce its crystalinity and make the fermentable sugars accessible to the fermenting organisms. The methods vary greatly in cost, complexity and several other factors. Different biomass types need varying degrees of pretreatments, depending on their chemical composition and complexity. During many pretreatment methods some chemicals are formed which can inhibit the fermentation or be toxic to the micro-organisms used. Therefore detoxification is often needed before the fermentation step can begin. Pretreatments are of various types such as biological, chemical, physical, physical-chemical and enzymatic.

2.3.1. Biological

Biological pretreatments have low energy requirements and use mild environmental conditions. The main types are fungal pretreatment and bioorganosolv pretreatments. Brown-, white- and soft-rot fungi are used in fungal pretreatment because they produce different types of cellulases, hemicellulases and lignin-degrading enzymes. Brown-rot

fungi degrade cellulose. White- and soft-rot fungi degrade cellulose and lignin. These are mostly used on corn stover and wheat straw but the degradation is very slow (Sun & Cheng, 2002) (Tengerdy & Szakacs, 2003). In bioorganosolv pretreatment, the fungi *Ceripoiopsis subvermispora* is applied for 2-8 weeks to decompose the lignin network and then it is followed by ethanolysis at 140-200°C for 2 hours. The biological pretreatment can save up to 15% of the electricity needed for the ethanolysis and the ethanol can be reused to spare the environment. This process has been used on Beech wood (Itoh, et al., 2003). The fact that biological pretreatments take such long time limits their industrial applications.

2.3.2. Chemical

Chemical pretreatments are many, such as ozonolysis, dilute-acid hydrolysis, concentrated-acid hydrolysis, alkaline hydrolysis, oxidative delignification, wet oxidation and organosolv process (Lynd, et al., 2002) (Sun & Cheng, 2002) (Cuzens & Miller, 1997) (Varga, et al., 2004). The acids most commonly used are sulfuric acid (H_2SO_4) and hydrocloride acid (HCl), concentrated or diluted. Use of dilute acids is more popular since it is cheaper in terms of equipment maintainance. Using dilute-acid pretreatment at low temperatures (121°C) avoids the degradation of sugars to furfural and hydroxymethylfurfural (HMF), but the sugars yields are lower (Saha, et al., 2005b). Physical-chemical methods are considered more effective than physical pretreatments. In this category are e.g. steam explosion, liquid hot water (LHV), ammonia fiber explosion (AFEX) and CO_2 explosion (Lynd, et al., 2002) (Sun & Cheng, 2002). The most widely studied methods are dilute acid pretreatment along with steam explosion. The main advantage of dilute acid pretreatment over steam explosion according to Lynd and coworkers is the higher recovery of sugars derived from hemicellulose (Lynd, et al., 2002).

Liquid hot water or thermohydrolysis is considered one of the most promising pretreatments. Under optimal conditions it is comparable to dilute acid pretreatment but without the addition of acids (Laser, et al., 2002). Generation of inhibitors is lower and recovery of pentoses is higher. However, solid load is much less than for steam explosion which is usually greater than 50% (Sánchez & Cardona, 2008).

2.3.3. Enzymatic

The enzymes used in starch hydrolysis are called amylases. The enzyme α-amylase is and *endo* hydrolase (cuts within the polymer), which hydrolyses and α-glycosidic bond and forms mainly maltose (disaccharide) and maltotriose. The enzyme β-amylase is an *exo* hydrolase (cuts sugar units at the end of the polymer), which splits the chain from the non-reducing end and releases maltose. When hydrolysing amylopectin the enzyme is unable to pass the 1,6-branch point, which results in the formation of glucose and a polysaccharide called limit dextrin. Pullulanases or γ-amylases are also exo enzymes,but they cleave both α-1,4 and α-1,6 linkages, resulting in glucose only.

Most enzymes used in hydrolysis of lignocellulosic biomass are optained from fungi, such as *Trichoderma reseei* and *Aspergillus niger*. There are three types of cellulases: cellobiohydrolases, endoglucanases and β-glucosidases. Cellobiohydrolases are *exo* enzymes which gradually decrease the polymerization degree, releasing cellobiose as the product. Cellobiohydrolase I prefers the reducing end and cellobiohydrolase II prefers the nonreducing end of microcrystalline cellulose. Endoglucanases are *endo* enzymes and they cause rapid reduction of polymerization by cutting the polymer into smaller chains, they prefer amorphous cellulose. Other *exo* enzymes are β-glucosidases which hydrolyse the cellobiose to glucose. Enzymatic hydrolysis is slower than chemical hydrolysis but it has the advantage of not degrading the glucose formed and thereby not forming any toxic and inhibitory compounds (Sánchez & Cardona, 2008).

2.3.4. Physical

Pure physical methods can be divided into two categories, mechanical comminution and pyrolysis (Sun & Cheng, 2002). Mechanical comminution is chipping, grinding, milling and any form of physical treatment to decrease the size of the biomass particles. This reduction in size increases the biomass surface, and thus facilitates access for cellulases. Pyrolysis is carried out by heating the biomass to temperatures above 300°C without oxygen and then cooling and condensing the residues. Pyrolysis forms char and volatile products, which can further undergo mild dilute-acid hydrolysis to produce reducing sugars.

2.3.5. Toxic compounds

As previously mentioned, during pretreatment, many inhibitory and toxic compound can be formed. These compounds are formed by degradation of sugars, lignin, lignosellulose and corrosion of the equipment (heavy metal ions). The inhibitory compounds formed belong to various categories such as phenols, furans and acids. From degradation of xylose (a pentose), furfural is formed (Adams & Voorhees, 1941):

$C_5H_{10}O_5 \rightarrow C_5H_4O_2 + 3\ H_2O$

From hexoses, hydroxymethylfurfural (HMF) is formed (Román-Leshkow, Chheda, & Dumesic, 2006):

$C_6H_{12}O_6 \rightarrow C_6H_6O_3 + 3\ H_2O$

From lignin and lignocellulose the inhibitors are aromatics, phenols and aldehydes.
These compound inhibit or slow down the fermentation by preventing the organisms used from growing (multiplying) or by killing them (Sánchez & Cardona, 2008) (Román-Leshkow, Chheda, & Dumesic, 2006).

2.3.6. Detoxification

When inhibitors and toxic compounds are formed, some form of detoxification methods are needed before the fermentation can be carried out. Detoxification methods can be divided into physical, chemical and biological methods. Physical detoxification includes evaporation, extraction and adsorption. Chemical detoxification is done by neutralization, alkaline detoxification (overliming), combined alkaline detoxification or ionic exchange. Biological methods are either enzymatic (using laccase/phenol oxidase and lignin peroxidase) or microbial (using *Trichoderma reesei* and other fungi) (Sánchez & Cardona, 2008).

2.4. Production of bioethanol

After pretreatments of the biomass, and possibly detoxification processes, the resulting hydrolysates are ready for fermentation. The final step involves distillation and condensation. Ethanol production for consumption has a very long history, in fact it has

been dated as far back as 7000 B.C. in China (McGovern, et al., 2004). Ethanol has not only been used in alcoholic beverages, is also useful in cleaning agents, cosmetics and antifreeze. In addition to the use as fuel supplement, ethanol is also used in the chemical industry, e.g. as feedstock for the synthesis of several chemicals like acetic acid and acetaldehyde (Kosaric & Vardar-Sukan, 2001)

2.4.3. Fermentation

Energy harvesting by organisms goes mainly by three ways: photosynthesis; respiration and fermentation. The aerobic degradation of biomass and organic substances is similar to the anaerobic degradation, the major difference is the presence of oxygen. For instance glucose is degraded by 10 steps (glycolysis) to pyruvate in both the presence and absence of oxygen.

Organisms store the energy from respiration or fermentation by forming adenosine triphosphate (ATP). The formation of ATP is endergonic and therefore needs energy from exergonic reactions. These reactions are carried out in the form of oxidizing the (organic) reactants forming an oxidized intermediate and reduced cofactor (like NADH and $FADH_2$), and by re-oxidizing the cofactors by the reduction of the product, hence the reduced end-products are formed (Fig. 2.7). The oxidation of the cofactors is carried out either by respiration, where oxygen is involved, or by fermentation (anaerobic) (Madigan, Martinko, & Parker, 2003).

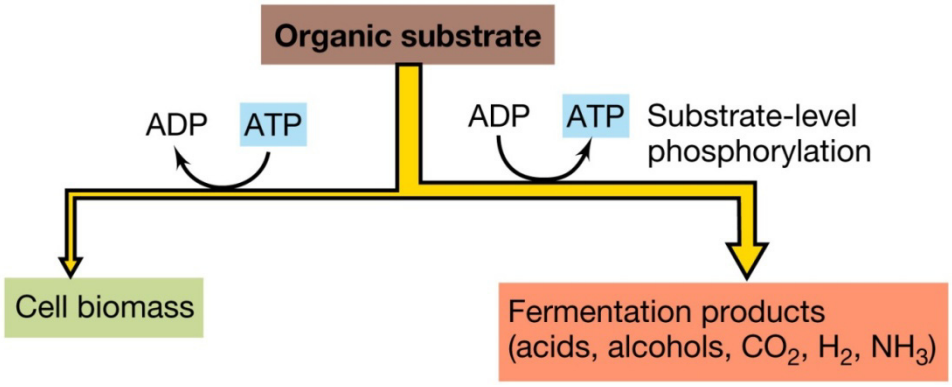

Figure 2.5. Fermentation pathways of organic material (Madigan, Martinko, & Parker, 2003)

Figure 2.5 shows that organisms always requires energy in the form of ATP for growth from a certain carbon source. This means that during degradation of a substrate a substantial part of its energy is used for energy production and a 100% yield of a

product can never be obtained. The formation of reduced end-products (such as ethanol) is due to the lack of oxygen as a final electron acceptor and the organisms need to regenerate the oxidized coenzymes. This formation of ATP by this route is called substrate level phosphorylation (SLP), whereas in respiration the generation of ATP is by oxidative phosphorylation and the reduced cofactors are re-oxidized by the proton motive force (PMF) (Madigan, Martinko, & Parker, 2003).

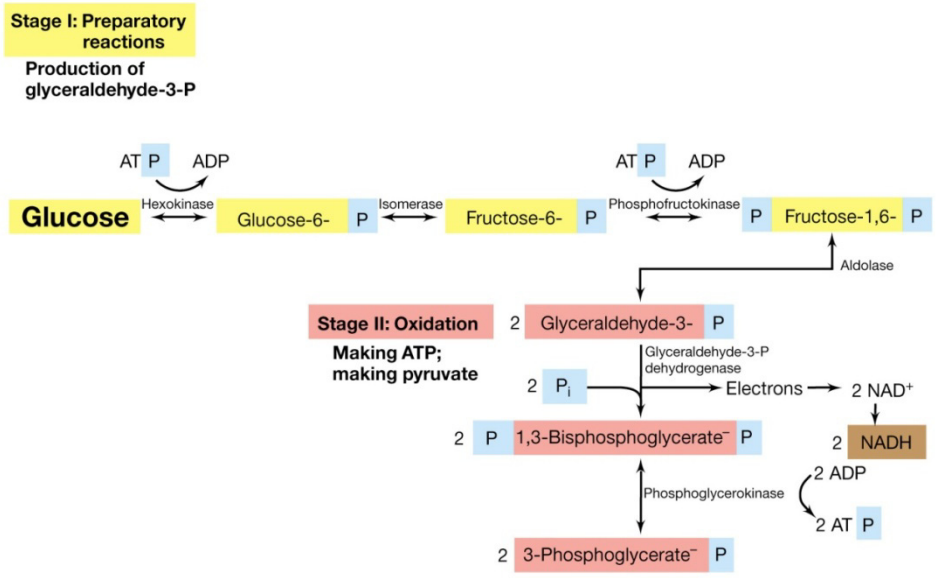

Figure 2.6. The first steps of the glycolysis, preparation and oxidating steps (Madigan, Martinko, & Parker, 2003)

The preparatory steps of the glycolysis utilize two ATP's when converting glucose to fructose-1,6-diphosphate, which then is oxidized in several steps and 4 ATP's are formed together with two molecules of pyruvate and NADH (Fig. 2.6). Under anaerobic conditions pyruvate is reduced to various end products (Fig. 2.7).

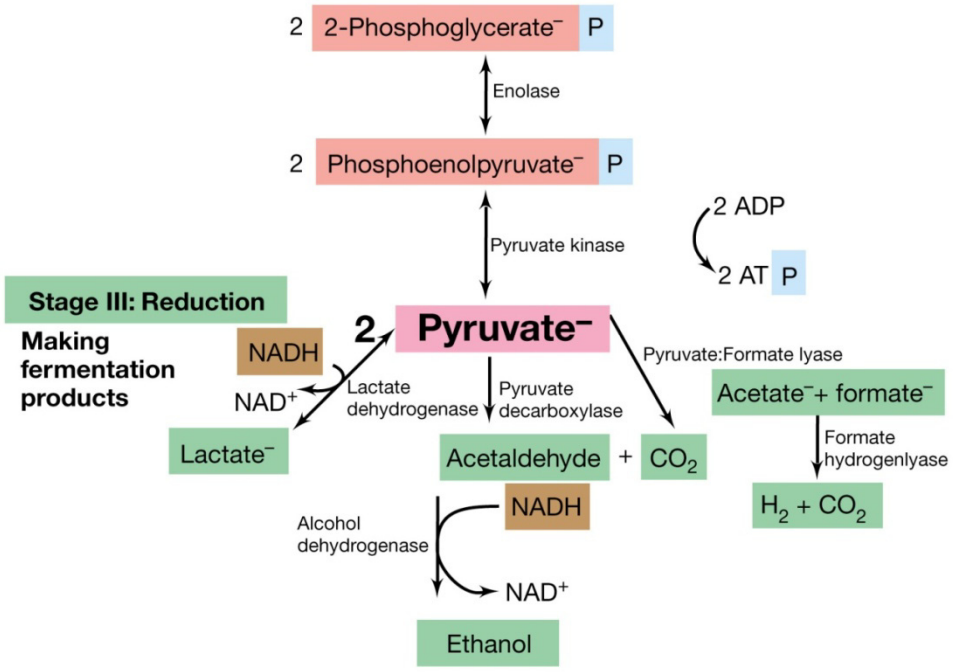

Figure 2.7. Reducing steps of the glycolysis (Madigan, Martinko, & Parker, 2003)

Figure 2.7 shows how pyruvate can be reduced to several end products, such as acetate, hydrogen, ethanol and lactate. Theoretical maximum yield of ethanol varies greatly with the various co-products that are often produced.

I. $1.0\ C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$

II. $1.0\ C_6H_{12}O_6 + H_2O \rightarrow 1\ C_2H_5OH + 1\ CH_3COOH + 2H_2 + 2CO_2$

III. $C_6H_{12}O_6 \rightarrow x\ C_2H_5OH$ + y other end-products + $z\ CO_2 + w\ H_2$

- x, y, z, w stand for different stoichiometries

Yeast have very high ethanol yields or close to 2 moles of ethanol for every mole of hexose (equation I). Some of the sugar is also converted to biomass which lowers the yield to 1.80 – 1.86 mol-EtOH/mol-glucose (90 - 93% of theoretical maximum). No hydrogen is produced by yeasts. The sugars are degraded through the common Embden-Meyerhof pathway (EM) but *Zymonomonas mobilis*, a Gram-negative bacterium is though known to produce up to 1.94 mol-EtOH/mol-glucose by the Entner-Deudoroff pathway (ED) (Ingledew, 1999) (Sprenger, 1996). The reason for exceptionally high yields with this bacterium is caused by less biomass produced but the bacterium

produces only 1 mol ATP/mol-glucose compared to 2 moles by EM pathway. Most bacteria however use heterofermentative pathways for ethanol production (equations II and III), i.e. they produce not only ethanol but various other end products like acetate, lactate, butyrate and formic acid (Wiegel, 1980). This leads to substantially lower yields of ethanol from hexose fermentation and can vary a lot.

2.4.4. First generation ethanol production

About 79% of ethanol produced in Brazil is from fresh sugar cane juice but the rest is from cane molasses which are a by-product of sugar mills (Wilkie, Riedesel, & Owens, 2000) which are e.g. the main feedstock in India Cane molasses are the main feedstock in India (Ghosh & Ghose, 2003). Starch needs to be hydrolyzed to become fermentable. This has traditionally been done chemically by acids but enzymes are more specific and need milder conditions which has facilitated their takeover of the process. The enzyme α-amylase is obtained from thermoresistant bacteria such as *Bacillus licheniformis* or from engineered strains of *Escherichia coli* or *Bacillus subtilis* for the first step of hydrolysis of starch, liquification. After the liquification is the saccharification step where glucoamylases are used from *Aspergillus niger* or *Rhizopus* species. When corn is used, it is milled to extract the starch. This can be done either by wet milling or dry milling. In wet-milling the corn grain is separated into its components, starch is converted to ethanol and the remaining components are sold as co-products. In dry milling the whole grain enters the process and the remaining nutrients (not used in the fermentation) are concentrated and sold as animal feed as DDGS (Dried Distiller's Grains with solubles) (Sánchez & Cardona, 2008). Ethanol can also be produced from wheat, by similar processes to that of corn (Soni, Kaur, & Gupta, 2003) (Wang, et al., 1999). Ethanol production from cassava has gained increased attention especially in tropical areas due to its abundancy. In the process either the whole cassava tuber can be used or the extracted starch, similar to corn. It has also been suggested that the cassava can be processed to wheat or chips before liquidization and saccharification (López-Ulibarri & Hall, 1997) (Sriroth, Lamchaiyaphum, & Piyachomkwan, 2007). Other starch rich biomass contributing to ethanol production are rye, barley, triticale and sorghum (Wang, et al., 1997) (Zhan, et al., 2003).

2.4.4.1. Microorganisms

The most employed micro-organism in ethanol production is the baker's yeast *Saccharomyces cerevisiae* because it can hydrolyze sucrose into glucose and fructose, two easily fermentable hexoses (Sánchez & Cardona, 2008). Other yeasts are also well known in ethanol production, such as *Schizosaccharomyces pombe* which has high tolerance for osmotic pressure and high solids content (Bullock, 2002) (Goyes & Bolanos, 2005). *Zymomonas mobilis* is a mesophilic bacterium which has very promising attributes considering ethanol production. It has a very low energy efficiency which results in a higher ethanol yield, up to 97% of theroretical maximum. Its disadvantages are its narrow substance range, but it can only utilize glucose, fructose and sucrose (Claassen, et al., 1999), and the formation of levan (poly-fructose), which increases the viscosity of the medium and of sorbitol during fructose fermentation, which decreases the efficiency of ethanol formation (Lee & Huang, 2000).

To be suitable for ethanol production the micro-organisms need to meet certain criteria (Dien, Cotta, & Jeffries, 2003):

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

Obviously, no one organism has these abilities naturally. Yeast can have some of them, such as simple nutrient requirements, high ethanol tolerance and productivity along with minimal by-product formation. Bacteria have other, such as broad substrate range and high ethanol productivity. Efforts have been made to add more of these abilities to some organisms by genetic engineering, and several have been successful (Dien, Cotta, & Jeffries, 2003) (Wood & Ingram, 1992).

2.4.5. Second generation ethanol production

Many types of lignocellulosic biomass have been tested for ethanol production and they can generally be divided into six groups:

1. Crop residues: cane bagasse, corn stover, wheat straw, rice straw, rice hulls, barley straw, sweet sorghum bagasse, olive stones and pulp.
2. Hardwood: aspen, poplar.
3. Softwood: pine, spruce.
4. Cellulose wastes: newsprint, waste office paper, recycled paper sludge.
5. Herbaceous biomass: alfalfa hay, switchgrass, red canary grass, coastal Bermudagrass, thiothy grass.
6. Municipal solid wastes (MSW).

The main limiting factor regarding industrializing ethanol production from lignocellulosic biomass is the higher degree of complexity related to the composition of the resource (Sánchez & Cardona, 2008). This complexity calls for pretreatments, but those do not fit all biomass types equally well. An ethanol production plant would therefore need to rely on only one or few types of biomass for its production.

2.4.5.1. SHF

Separate hydrolysis and saccharification (SHF) is the classic configuration for fermenting biomass hydrolyzates. It involves a sequential process where the hydrolysis of the cellulose and the fermentation are carried out in different units. In this process the solid fraction of pretreated lignocellulosic biomass, which contains the cellulose in a form accessible to acids or enzymes, undergoes hydrolysis (saccharification). When the hydrolysis is completed the result is cellulose hydrolysate containing free fermentable sugars. This hydrolysate is then fermented in a separate unit. One of the main advantages of SHF is that each step can be carried out at its optimum conditions. Important factors to consider for the saccharification step are reaction time, temperature, pH, enzyme dosage and substrate load (Sánchez & Cardona, 2008). Hari Krishna and coworkers experimented with pretreated sugar cane leaves (by overliming) to find the optimal conditions. They achieved 65-70% cellulose conversion at 50°C and pH 4.5. An enzyme load of 40 FPU/g cellulose resulted 87% conversion and is economically justifiable. Solids loads should be 10%, to avoid mixing difficulties and accumulation of inhibitors (Hari Krishna, et al., 1998). Tengborg and coworkers found that steam-pretreated spruce also needed high loadings of enzymes to achieve over 70% cellulose conversions, due to the less degradability of the softwood (Tengborg, Galbe, & Zacchi, 2001). Titmas patented bioethanol production from the cellulosic portion of MSW in 1999 (Titmas, 1999). Nguyen and coworkers employed a mixed solids waste for

producing ethanol by SHF using yeasts. They recycled the hydrolyzing enzymes through microfiltration and ultrafiltration and achieved 90% cellulose hydrolysis at a net enzyme loading of 10 FPU/g cellulose (Nguyen, et al., 1999).

2.4.5.2. SSF

Simultaneous saccharification and fermentation (SSF) is the alternative to SHF. In SSF processes the hydrolysis and fermentation are carried out in a single unit, at the same time. Figure 2.8 shows a schematic representation of an SSF process. By SSF higher ethanol yields and less energy consumption have been obtained. Since cellulases and microorganisms are added to the same unit in this process, the glucose formed by hydrolysis can be immediately consumed and fermented to ethanol. This neutralizes the inhibition effect of glucose on the cellulases. More dilute media is required though, to reach suitable rheological properties, and that makes final product concentration low. Another disadvantage is the compromise required between the optimal conditions for the enzymes and for the microorganims. In addition, higher dosage of enzymes are needed, which is positive for the cellulose conversion but negative for the production costs. Surfactants have been proposed as a solution to these high dosages (Sánchez & Cardona, 2008). Tween-20 has been tested with this regard and resulted in 8% higher ethanol yield, 50% reduction of enzymes dosage, increase of enzyme activity and decrease in fermentation time (Alkasrawi, et al., 2003). Other investigations have however showed only marginal increases in saccharification (3,5%) (Saha, et al., 2005b)

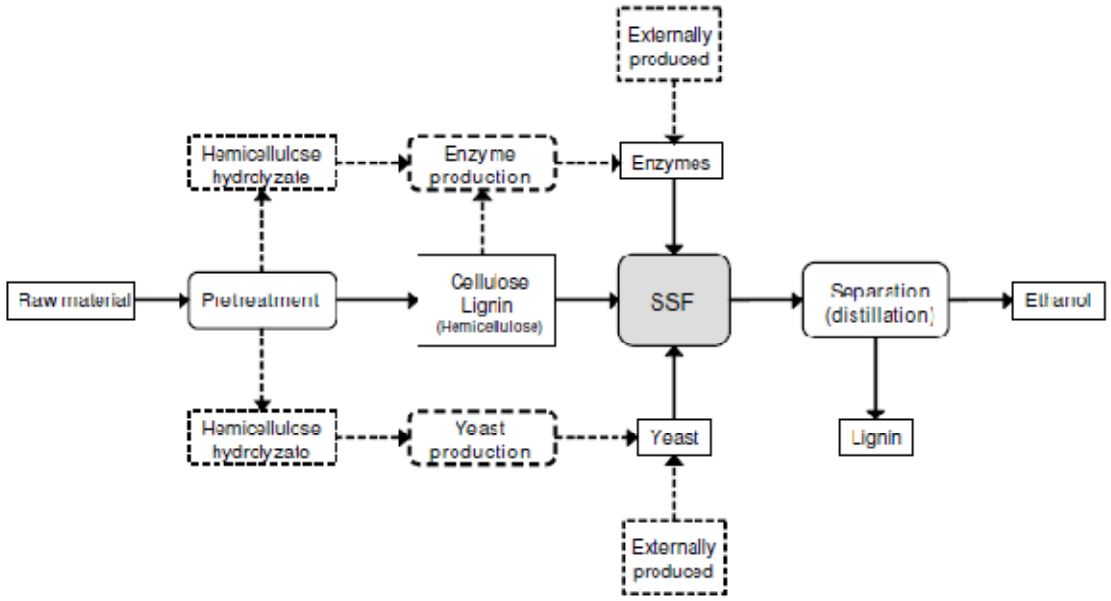

Figure 2.8. A schematic representation of an SSF process (Olofsson, Bertilsson, & Lidén, 2008)

Hari Krishna and coworkers also evaluated the optimal conditions for SSF as they did for SHF, using sugar cane leaves. They found the optimum temperature to be 40°C and pH 5.1 for 3 day cultivation, using 15% substrate load and enzyme load of 100 FPU/g cellulose, which is quite high (Hari Krisha, et al., 1998). Stenberg and coworkers used spruce with 5% substrate loading and doubled the productivity related to SHF, with an enzyme load in the range 5-32 FPU/g cellulose (Stenberg, et al., 2000).

2.5. Thermophiles

Thermophilic bacteria can be divided into three categories: thermophilic bacteria which live at at 45- 68°C with optimum around 60°C; extreme thermophilic bacteria which live at 65-95°C with optimum around 88°C and pyrophilic bacteria which live at even higher temperatures such as *Pyrolobus fumarii* which has optimum at 106°C and can survive up to 115°C. These bacteria do not grow below 60°C (Huber & Setter, 1998). One of the major disadvantages of using thermophiles for ethanol production is their relatively low tolerance towards ethanol. Wild type bacteria usually have tolerance in the range of 2 to 4% (vol/vol). While yeast can tolerate up to 12% (vol/vol). This is one of the main reasons that yeast is still widely used today. Ethanol tolerance of thermophiles can be increased either by genetical engineering, selective mutation or

environmental pressure (adjustment to higher concentrations) (Lovitt, Shen, & Zeikus, 1988) (Method for culturing microorganisms). Another factor in ethanol production to consider is the ethanol formation rate, this has to be competitive with the yeasts in current use (Dien, Cotta, & Jeffries, 2003).

2.5.1. Geothermal areas

Thermophiles can be found in various places and grow anywhere where there is enough heat. Natural habitats therefore include geothermal sites associated with volcanic activity. Terrestrial sites are of two main types i.e. solfatara fields with sulphuric and acidic soils, acidic hot springs and boiling mud pots, while the other has fresh-water hot springs and geysers with neutral-to-alkaline pHs. In Iceland geothermal areas cover over 500 km^2 which is about 0.5% of the countries surface (Kristjansson & Stetter, 1991). There are around 30 solfatara fields in Iceland and 250 low heat fields with over 600 geysers and pools (Hveravefsíðan, 2005).

2.5.2. Microbial flora of Icelandic geothermal areas

As geothermal areas vary greatly in conditions like temperature and acidity, their microbial flora varies accordingly. Many species of anaerobic archaea have been isolated from the clayish hot-springs in the solfatara fields in Iceland. Optimal conditions for these microbes are in temperature range of 85-95°C and pH below 4. They can utilize organic matter and use sulphur as electron donor. They can also form hydrogen sulphide (H_2S). One species of methanogens has also been isolated from these zones, *Methanothermus fervidus.* Other bacteria isolated from these areas are *Thermoproteus, Thermofilum* and *Desulfurococcus*. The conditions in these hot-springs can be similar to those in continuous reactors because of vast and constant flow of gases bringing nutrition for chemolithotrophic bacteria. Microorganisms can be high in numbers, as much as 100 million per milliliter (10^8/ml). The low temperature zones have very different conditions to those in the solfatara fields. Hot-springs are mainly alkaline (pH 8-10) and the temperature range is from 20 to 100°C. Formation of silica is prominent around openings of these springs, and algae and high quantities of bacteria can be found there. Common species in hot-springs in the range of 50-85°C are *Chloroflexus*, a filamentous species that practices anaerobic photosynthesis, *Thermus*, which are Gram-negative rods, often filamentous. Hydrogen oxidizing, sulphate reducing and methane producing bacteria have also been isolated from these zones

(Kristjansson & Alfreðsson, 1986). Hydrogen and ethanol producers have also been isolated and fall into the genuses of *Clostridium* and *Thermoanaerobacterium* (Orlygsson & Baldursson, 2007). The strain *Thermoanerobacter mathranii* is an ethanol producer isolated from a 70°C hot, alkaline (pH 8.5) hot-spring in Iceland (Larsen, Nielsen, & Ahring, 1997). Cellulose and hemicellulose degrading bacteria have also been found in these areas (Mathrani, et al., 1993).

2.5.3. Anaerobic bacteria

As stated earlier anaerobic bacteria do not use oxygen as electron acceptor but produce intead various reduced end products to oxidize NADH to NAD^+. Among end products are acetate, ethanol, butyrate, lactate and hydrogen (Madigan, Martinko, & Parker, 2003). This range of end products pose a problem if the main aim is to produce ethanol because other end products need to be removed. This problem can be solved with genetic engineering by cutting the unwanted product's pathway.

There are many bacteria that are known to produce ethanol at high temperatures. The focus will be on the following genera: *Clostridia, Caloramator, Caldicellulosiruptor, Thermoanaerobacter, Thermoanaerobacterium, Thermoanaerobium* and *Thermobrachium*.

2.5.3.1. *Clostridium*

The genus *Clostridium* belongs to the family Clostridiaceae, order Clostridiales, class Clostridia and phylum Firmicutes. Before the use of 16S rRNA analysis for classification of prokaryotes the genus *Clostridium* was defined as bacteria that were non-sulfate reducing, spore forming and anaerobic with Gram positive cell wall. The family Clostridiaceae contains the genus *Clostridium* (sensu strict), as well as many other genera, e.g. *Acetivibrio, Acidaminobacter, Anaerobacter, Caloramator* and more (Rainey, Tanner, & Wiegel, 2006). The reason for these additional genera in the family Clostridiaceae is often based upon the fact that the type species of these genera are often phylogenetically related to misclassified species of the genus *Clostridium*. However, many of these genera fall outside what is considered to be the "true Clostridiaceae" family and are now regarded to belong to other families within the low G+C Gram-positive phylum. In the last ten years our view of bacterial classification has greatly changed because of the use of 16S rRNA analysis for classification of bacteria. The phylogenetic analysis of Collins and co-workers was the first time when a substantial

number of *Clostridium* species were compared both within species belonging to the genus and to related taxa (Collins, et al., 1994). The fact that the species of the genus *Clostridium* did not form a monophyletic group has been shown in many studies (Tanner, et al., 1981)(Rainey, et al., 1993) (Hutson, Thompson, & Collins, 1993)(Lawson, et al., 1993) (Rainey & Stackebrandt, 1993) (Willems, Amat-Marco, & Collins, 1996). These investigations lead to the conclusion that more than half of the species currently assigned to the genus *Clostridium* are in fact not closely related to the type species *C. butyricum* and should therefore not be included in the newly defined genus *Clostridium*. The genus *Clostridium* comprises more than 150 validly described species (see (Euzéby, 2009) the List of Bacterial Names with Standing in Nomenclature). On the basis of 16S rRNA only 73 of these species fall within the radiation of the type species of the genus *Clostridium, C. butyricum* and the cluster I as defined by Collins and coworkers (Collins, et al., 1994). The remaining species fall within the various phylogenetic clusters throughout the low G+C Gram-positive phylum. Additionally, the genus *Clostridium* (sensu strict, cluster I) has members of other valid genera and in two cases the type species of validly described genus clustering within its radiation (*Anaerobacter* and *Eubacterium*). The genus *Clostridium* contains 213 species, but not all are thermophiles (GmbH, 2004). *Clostridium thermohydrosulfuricum* is known to produce 1 mol ethanol per mol glucose (Wiegel, 1980).

Several of the cultures isolated in present study were classified within several clusters of *Clostridium*.

2.5.3.2. *Caloramator*

There are six species that belong to the genus *Caloramator*, i.e. *C. fervidus, C. indicus, C. proteoclasticus, C. coolhaasii, C. viterbensis* and *C. australicus*. The genus belongs to the order Clostridiales, the class Bacilli and the pylum Firmicutales. *Caloramator* is from the latin noun *calor* which means heat and *amator* which means lover, so *Caloramator* means heat lover (Collins, et al., 1994). The genus forms a distinct cluster within the Gram-positive phylum and is closely related to *Thermobrachium celere.* Cells are rod shaped and stain gram variable, but microscopy reveals a gram positive wall structure. Endospores are formed in some species. All species belonging to this genus are thermophilic and obligate anaerobes. Temperature optima are within the range of 55° - 68°C, while growth occurs at 30° – 80°C. pH optima are with in the range of 6 -

7.5 although growth occurs at a range from 5.5 to 9.5. *Caloramator* species are not so much known for ethanol production, although most of them form some amounts along with acetate, carbon dioxide, hydrogen and lactate. *C. proteoclasticus* forms formate rather than carbon dioxide (Tarlera, et al., 1997). *C. coolhaasii* also forms small amounts of propionate (Plugge, Zoetendal, & Stams, 2000). *C. viterbensis* stands out as a glycerol fermentor with acetate and 1,3-propanediol as sole end products (Seyfried, et al., 2002). The species are isolated from various sources in different continents, e.g. from hot water in India, hot springs in Italy and the Great Artesian Basin of Australia (Chrisostomos, et al., 1996)(Seyfried, et al., 2002). *C. australicus* is the newest addition to the genus and makes only ethanol and acetate during fermentation of glucose (Ogg & Patel, 2009). Type species is *C. fervidus* (Patel, et al., 1987) (Collins, et al., 1994).

Several of the cultures isolated in present study were classified within the genus *Caloramator.*

2.5.3.3. *Caldicellulosiruptor*

There are seven species that belong to the genus *Caldicellulosiruptor*, i.e. *C. saccharolyticus, C. lactoaceticus, C. ownsensis, C. kristjanssonii, C. acetigenus, C. hydrothermalis* and *C. kronotskyensis*. The genus belongs to the family Thermoanaerobacteriaceae, the order Thermoanaerobacterales, the class Clostridia and the pylum Firmicutes. The name *Caldicellulosiruptor* implies that the species degrade cellulose. Cells are rod shaped and stain gram variable, but microscopy reveals a gram positive wall structure. Spores are not formed in these species. All species belonging to this genus are extremely thermophilic and obligate anaerobes. Temperature optima are within the range of 65° - 78°C, while growth occurs at 45° – 82°C. pH optima are with in the range of 7.0 - 7.5 although growth occurs at a range from 5.2 to 9.0 *Caldicellulosiruptor* species are not so much known for ethanol production, although some of them form some amounts along with acetate, carbon dioxide, hydrogen and lactate. *Caldicellulosiruptor* species are known for coupling cellulose degradation to growth (Rainey, et al., 1994). *C. acetigenus* does not however utilize cellulose in the form of Whatman paper, but its ability to hydrolyze CMC justifies its reclassification from *Thermoanaerobium* (Onyenwoke, et al., 2006) (Nielsen, Mathrani, & Ahring, 1993). The species are isolated from various places e.g. terrestrial hot springs in Kamchatka, east Russia, Owens lake in California, USA and one from an Icelandic hot spring, named after an Icelandic professor Dr. Jakob Kristjánsson (Miroshnichenko, et

al., 2008) (Huang, et al., 1998) (Bredholt, et al., 1999). *C. kristjanssonii* produces mainly acetate and hydrogen from cellulose, xylan and pectin (Bredholt, et al., 1999). *C. lactoaceticus* produces mainly lactate rather than acetate and grows only on limitied number of carbohydrates (Mladenovska, Mathrani, & Ahring, 1995). Type species is *C. saccharolyticus,* described in 1994 by Rainey and coworkers (Rainey, et al., 1994).

Several of the cultures isolated in present study were classified within the genus *Caldicellulosiruptor.*

2.5.3.4. *Thermoanaerobacter*

The genus *Thermoanerobacter* contains fifteen validly described species, i.e. *T. acetoethylicus, T. brockii, T. cellulolyticus, T. ethanolicus, T. italicus, T. kivui, T. mathranii, T. pseudethanolicus, T. siderophilus, T. subterraneus, T. tengcogensis, T. thermocopriae, T. uzonensis, T. wiegelii* and *T. yonseiensis*. The genus belongs to the family Thermoanaerobacteriaceae, the order Thermoanaerobacterales, the class Clostridia and phylum Firmicutes. The name *Thermoanaerobacter* implies that the species are thermophilic and anaerobic bacteria. *Thermoanaerobacter* (and *Thermoanaerobacterium*) falls within the clusters V, VI and VII in phylogenetic interrelationships of *Clostridium* species (Collins, et al., 1994). The taxonomy of this group was revised by Lee and co-workers (Lee, et al., 1993). The genus *Thermoanaerobacter* was first described in 1981 and the name implies that the species are thermophilic, anaerobic bacteria (Wiegel & Ljungdahl, 1981). Bacteria within the genus stain Gram-variable but microscopy often reveals gram type positive cell wall. Sporulation is variable. Temperature optima are within the range of 55° - 75°C, while growth occurs at 32,5° – 85°C. The pH optima are with in the range of 5.8 - 8.5 although growth occurs at a range from 4.2 to 9.8. The G+C content of the DNA is 32 – 41 mol%. *Thermoanaerobacter* species are well known for ethanol production, as the type species *T. ethanolicus* suggests (Wiegel & Ljungdahl, 1981). *T. ethanolicus* is known to produce 1.8 mol ethanol per mol glucose (Wiegel, 1980).

Along with ethanol many produce acetate, lactate, hydrogen and carbon dioxide (Lee, et al., 1993) (Wiegel & Ljungdahl, 1981) (Larsen, Nielsen, & Ahring, 1997). For *T. tengcongesis* the main fermentation product is acetate (Xue, et al., 2001). *T. wiegelii* produces propionate in addition to ethanol, acetate, lactate and hydrogen (Cook, et al., 1996). *T. mathranii* is known to produce ethanol, acetate, propionate, hydrogen and

carbon dioxide from xylose (Larsen, Nielsen, & Ahring, 1997). The species have been isolated from various environments such as geothermal area in Kamchatka in Russia, Yellowstone national park in USA , sugar-beet juice and Italy, just to name a few (Wagner, et al., 2008) (Slobodkin, et al., 1999) (Lee, et al., 1993) (Onyenwoke, et al., 2007) (Carlier, Bonne, & Bedora-Faure, 2006) (Kozianowski, et al., 1997). *T. acetoethylicus* was reclassified from *Thermobacteroides* (Rainey & Stackebrandt, 1993), while others are being reclassified from *Thermoanaerobacter* to a new genus, *Caldanaerobacter* (Fardeau, et al., 2004) (Fardeau, et al., 2000) (Kim, et al., 2001). *Thermoanaerobacter keratinophilus* does not have a standing name in the nomenclature, but is nevertheless known to degrade wool and feathers, proving its possession of some desireble proteases (Riessen & Antranikian, 2001). The newest addition to this genus is *Thermoanaerobacter sulfurigignens*, which is known to reduce thiosulfate to elemental sulfur and tolerate high amounts of sulfite (Lee, et al., 2007).

Several of the cultures isolated in present study were classified within the genus *Thermoanaerobacter.*

2.5.3.5. *Thermoanaerobacterium*

There are eight species that belong to the genera *Thermoanaerobacterium*, i.e. *T. aotearoense, T. fijiensis, T. polysaccharolyticum, T. saccharolyticum, T. thermosaccharolyticum, T. thermosulfurigenes, T. xylanolyticum* and *T. zeae*. The genus belongs to the order Thermoanaerobacterales, the class Clostridia and the pylum Firmicutes. *Thermoanaerobacterium* is put together from the greek words, thermos (hot), an (not), aer (air) and bacterion which means a small rod, and means a rod which grows in the absence of air at high temperatures. Cells are straight rods and form long filaments. They are motile and peritrichous with gram negative cell wall and spores may be detected. Some species reduce thiosulfate to elemental sulfur, others to sulfide. All species belonging to this genus are thermophilic and obligate anaerobes. Temperature optima are within the range of 60° - 68°C, while growth occurs at 35° – 72°C. pH optima are with in the range of 5.2 – 6.8 although growth occurs at a range from 3.8 to 8.4. The G+C content of the DNA is 29 - 46 mol%. The main end products of carbohydrate fermentation are ethanol, acetate, lactate, hydrogen and carbon dioxide. Species have been isolated from various sources, such as geothermal sites in New Zealand, waste piles from canning factory in Illinois, USA and Fijian hot springs, to name a few (Liu, et al., 1996) (Cann, et al., 2001) (Lee, et al., 2008). Type species is

Thermoanaerobacterium thermosulfurigenes which is reclassified from *Clostridium* (Lee, et al., 1993). Due to constant development in this field and efforts to have accurate classifications, two *Thermoanaerobacterium* strains, i.e. *polysaccharolyticum* and *zeae* have recently been reclassified into a new genus along with a new isolate. The genus is *Caldanaerobius* and the type species is *C. fijiensis*, an inulin-degrading, ethanol producing thermophile (Lee, et al., 2008).

Several of the cultures isolated in present study were classified within the *Thermoanaerobacterium.*

2.5.3.6. *Thermoanaerobium*

The genera *Thermoanaerobium* contained five species, i.e. *T. acetigenum, T. brockii, T. crenophilum, T. lactoethylicum* and *T. olidum* according to the dsmz-database (GmbH, 2004). *Thermoanaerobium brockii* was effectively published in 1979 and validly in 1983, ten years later however it was reclassified as *Thermoanaerobacter*, and being the type species that made the remaining strains follow (Lee, et al., 1993) (Cayol, et al., 1995) (Zeikus, Hegge, & Anderson, 1979). *Thermoanaerobium acetigenum* was effectly published in 1993 and validly in 1994, the reclassification came in 2006 to *Caldicellulosiruptor* (Nielsen, Mathrani, & Ahring, 1993) (Onyenwoke, et al., 2006).

2.5.3.7. *Thermobrachium*

The genus *Thermobrachium* contains only one species i.e. *Thermobrachium celere* which belongs to the low-G+C-content, Gram type-positive *Bacillus-Clostridium* subphylum. This species is proteolytic, thermophilic obligate anaerobe. Cells are chemoorganoheterothrophs and proteolytic but not cellulolytic. Temperature optimum is 66°C, while growth occurs at 43° – 75°C. The pH optimum is 8.2 although growth occurs at a range from 5.4 to 9.5. The G+C content of the DNA is 30 – 31 mol%. The end products of glucose fermentation, in the presence of yeast extract, are ethanol, acetate, formate, hydrogen and carbon dioxide. This species produces almost equal amounts of acetate, formate, CO_2, and H_2. It does not produce valerate or butyrate and produces only trace amounts of lactate. In presence of yeast extract the strain utilizes glucose, sucrose, fructose galactose, and maltose. The species contains many strains however, which were isolated from various sources, such as sediment and water near a bathhouse in Ohinimutu, New Zealand, sediment and water in Toscani, Italy, Horse manure compost in Athens, Georgia, mesobiotic environments in Platte river, Buonos

Aires in Argentina, Elbe river in Germany and Mono lake in California, USA. The type strain is JW/YL-NZ35 (Engle, et al., 1996).

2.5.3.8. *Paenibacillus*

Several isolates in present study belong to the genus *Paenibacillus* which belongs to the order Bacillales, the class Bacilli and the phylum Firmicutes. *Paenibacillus* means almost Bacillus. Members of the genus *Paenibacillus* are facultative anaerobic, spore forming rods which stain gram variable. Most species are mesophiles but thermophilic strains have been reported (Wang, et al., 2008). Some of these bacteria excrete diverse assortments of extracellular polysaccharide hydrolyzing enzymes, including xylanases, cellulases, amylases, gelatinases, ureases and β-galactosidases (Velázquez, et al., 2004). The genus *Paenibacillus* contains 89 species, but very few are thermophiles but many bare names which indicate their polymer degrading abilities, e.g. *P. amylolyticus, P. cellulosilyticus* and *P. xylanilyticus* (Shida, et al., 1997) (Rivas, et al., 2006) (Rivas, et al., 2005). Temperature optima can be from 28 – 37 °C with the exception of *Paenibacillus sp.* which has optimum 50°C (Wang, et al., 2008). The pH optima are mostly neutrophilic and the G+C content of the DNA is in the range from 42 – 53 mol%. The type species is *P. polymyxa*. The genus was made be reclassification of some *Bacillus* strains (Ash, Priest, & Collins, 1993)

Several of the cultures isolated in present study were related to the *Paenibacillus.*

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4. Research of the present study

This project started in spring 2007 with the project BioEthanol at the University of Akureyri. A large bioprospecting survey on thermophilic anaerobic bacteria was performed by collecting samples from a well known geothermal area in Grensdalur, close to Hveragerði, in south west Iceland. More than 60 samples were collected and most of them were enriched on various carbon sources. After extensive enrichments and isolations, 60 samples were used as a base for manuscript I. Later on, the project was more directed towards the ability of the strains to produce ethanol (and hydrogen). Therefore the best strains (6) were investigated in detail concerning both phylogenetic and physiological characteristics. One strain was finally used for determining its ability to degrade sugars derived from various lignocellulosic material (manuscript II) together with another strain AK_{17}, already isolated at our laboratory. The other five strains were however, also investigated for their ethanol production capacity but are not part of the manuscripts included in present thesis. Therefore, below, data from these experiments are presented. The methodology and discussion is not included in this data, but is merely an extension of the kind of data that was aquired by the investigator.

Phylogenetic and physiological properties of five thermophilic, ethanol producing anaerobes, isolated from Icelandic geothermal areas.

After extensive isolation of thermophilic bacteria on various carbon substrates producing ethanol (manuscript I) it was decided to focus on six strains and investigate them in detail with respect to phylogeny and various physiological characteristics. These strains were 15-06-G, 25-07-C, 64-07-X, 66-07-P, 66-07-G, 67-07-P. One strain, 66-07-G is the main subject of manuscript II, but here data from experiments on the other five species are presented.

Phylogenetic studies of the bacteria

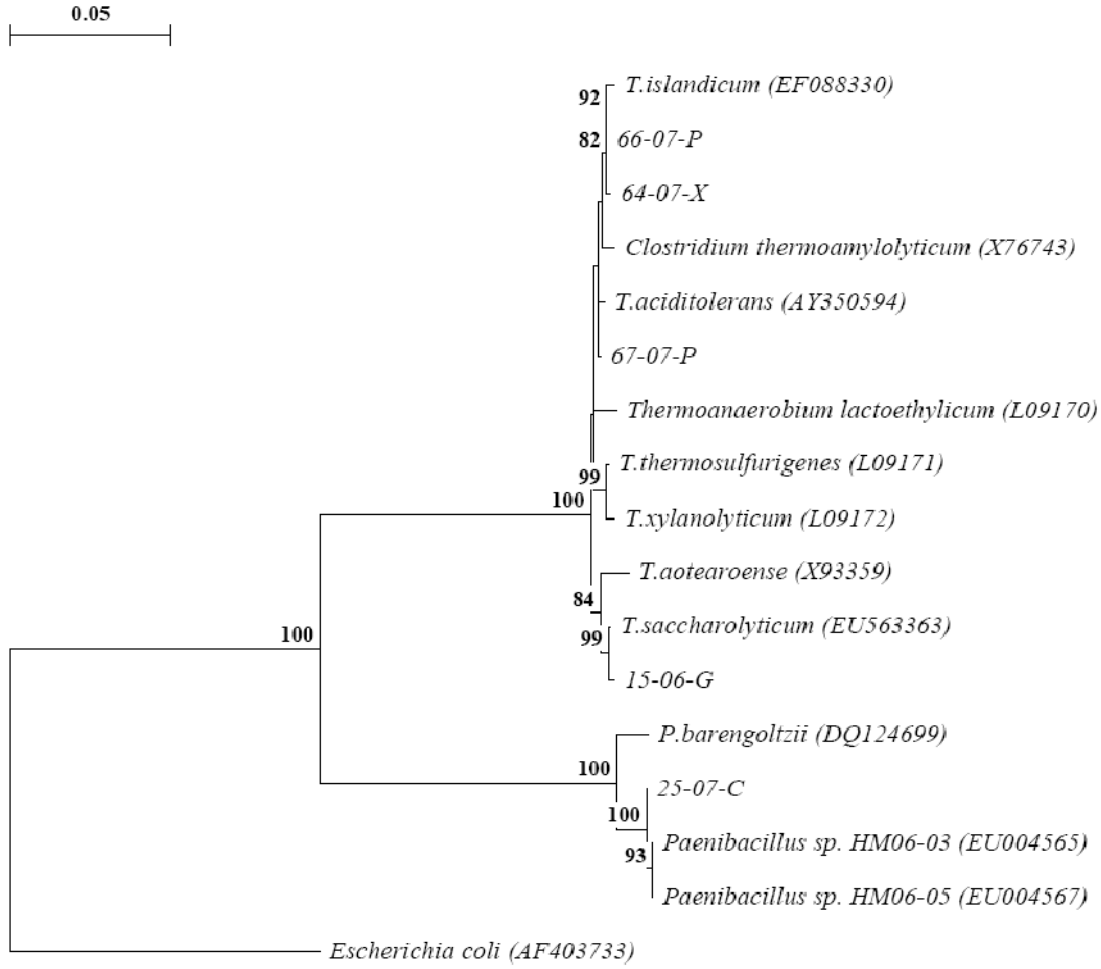


Figure 4.1. Phylogeny of the strains based on the 16S rRNA full sequences (≈1300 bp) for the five isolates. The phylogenetic tree was generated using a distance matrix and neighbor joining algorithms with 500 bootstraps. Only supported bootstrap values (>80%) are shown. Escherichia coli (AF403733) was selected as an out-group. The scale bar indicates 0.05 substitutions per nucleotide position.

Four of the strains belong to the genus *Thermoanaerobacterium* and one to *Paenibacillus*. The phylogenetic distance between the four *Thermoanaerobacterium* strains is very close (interrelated difference between 98.6 to 99.8%) when compared to each other (Fig. 4.1.). Their closest relatives are *Clostridium thermoamylolyticum* (99.1 to 99.8%) and *Thermoanaerobacterium aciditolerans* (99.0 to 99.6%). Strain 25-07-C was the only strain isolated on cellulose and is phylogenetically far away from the other

strains. 16S rRNA analysis reveals that this strain belongs to the genus *Paenibacillus* (Fig. 4.1.).

Isolations of bacterial strains – pH and temperature optimum

Tables 4.1. – 4.3. show the main characteristics of the strains concerning environmental conditions used for isolations, temperature and pH optimum and the names of the genera that are most closely related to them. The four strains were mainly isolated on polymeric carbohydrates but one on glucose (Table 4.1.).

Table 4.1. Carbon substrates used for isolation of five thermophilic bacteria strains. Also shown is the environmental factors used during enrichment and isolations and the closest phylogenetic genera.

Strain	Genus	Carbon source	T (°C)	pH
15-06-G	*Thermoanaerobacterium*	Glucose	60	7
25-07-C	*Paenibacillus*	Cellulose	50	7
64-07-X	*Thermoanaerobacterium*	Xylan	60	7
66-07-P	*Thermoanaerobacterium*	Pectine	60	7
67-07-P	*Thermoanaerobacterium*	Pectine	60	7

Table 4.2. Determination of T_{opt} for five thermophilic bacterial strains. Generation time and maximum optical density of strains is shown as well as isolation temperature.

Strain	Genus	Topt	Generation time (h)	OD max (600 nm)
15-06-G	*Thermoanaerobacterium*	65	1,58	1,31
25-07-C	*Paenibacillus*	50	0,71	1,82
64-07-X	*Thermoanaerobacterium*	60	1,01	1,24
66-07-P	*Thermoanaerobacterium*	60	0,96	1,47
67-07-P	*Thermoanaerobacterium*	60-65°C	1,11	1,44

Table 4.3. Determination of pH_{opt} for five thermophilic bacterial strains. Generation time and maximum optical density of strains is shown as well as isolation temperature .

Strain	Genus	pHopt	Generation time (h)	OD max (600 nm)
15-06-G	*Thermoanaerobacterium*	5	1,32	1,12
25-07-C	*Paenibacillus*	8	0,39	1,59
64-07-X	*Thermoanaerobacterium*	5	0,91	1,07
66-07-P	*Thermoanaerobacterium*	5	1,99	1,37
67-07-P	*Thermoanaerobacterium*	4.5-6.0	1,11	1,21

The strains were isolated at temperatures between 50 to 60°C. During isolations they were incubated at temperatures slightly below their natural environmental temperatures. Thus, it was not surprising that most of the strains had a similar temperature optimum as their isolation temperature (Tables 4.1. and 4.2.). The generation time for the strains at different temperatures varied from 0.71 h (strain 25-07-C) to 1.58 h (strain 15-06-G). The *Paenibacillus* (25-07-C) has the lowest T_{opt} (50°) but the four *Thermoanaerobacterium* strains grew best at between 60 – 65°C (Fig. 4.2.; Table 4.2.). Strikingly, all strains grew best at pH that differed from their native (environmental) pH's. Again, the fastest growing strain was 25-07-C but strain 66-07-P had the slowest growth rate (Fig. 4.3.; Table 4.3.). As for the temperature optimum there is a clear relationship between the phylogenetic status and the pH optimum. Most of the *Thermoanaerbacterium* strains have low pH optimum (pH 5.0 – 6.0) whereas the *Paenibacillus* strain had pH optimum at pH 8.0.

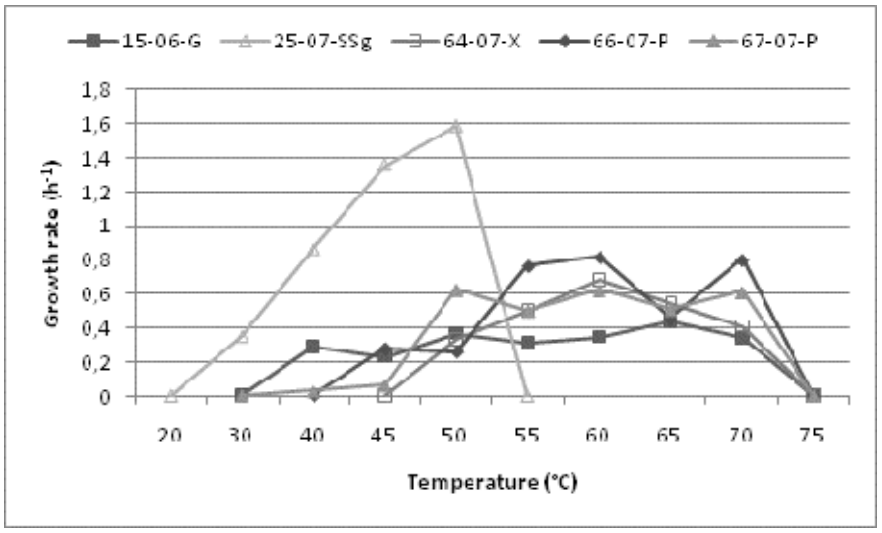


Figure 4.2. Temperature optimum for the five thermophilic bacteria isolated.

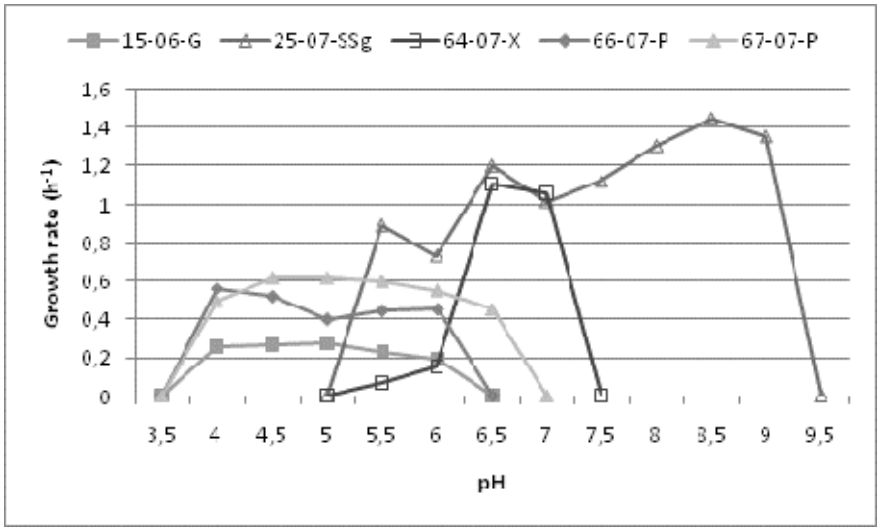


Figure 4.3. pH optimum for the five thermophilic bacteria isolated.

Physiological properties of strains

Ethanol tolerance. Table 4.4. shows the ethanol tolerance of the five strains. All strains showed ethanol tolerance up to 3.2% (v/v).

Table 4.4. Minimum inhibitory concentrations of ethanol for five thermophilic bacterial strains.

		% vol/vol							
Strain	Genus	0.0	0.2	0.4	0.8	1.6	3.2	6.4	8.0
15-06-Gl	*Thermoanaerobacterium*	+	+	+	+	+	+	-	-
25-07-C	*Paenibacillus*	+	+	+	+	+	+	-	-
64-07-X	*Thermoanaerobacterium*	+	+	+	+	+	+	-	-
66-07-P	*Thermoanaerobacterium*	+	+	+	+	+	+	-	-
67-07-P	*Thermoanaerobacterium*	+	+	+	+	+	+	-	-

Substrate inhibition. All five strains were incubated at various initial glucose concentrations varying from 5 to 400 mM (Fig. 4.4.A-E).

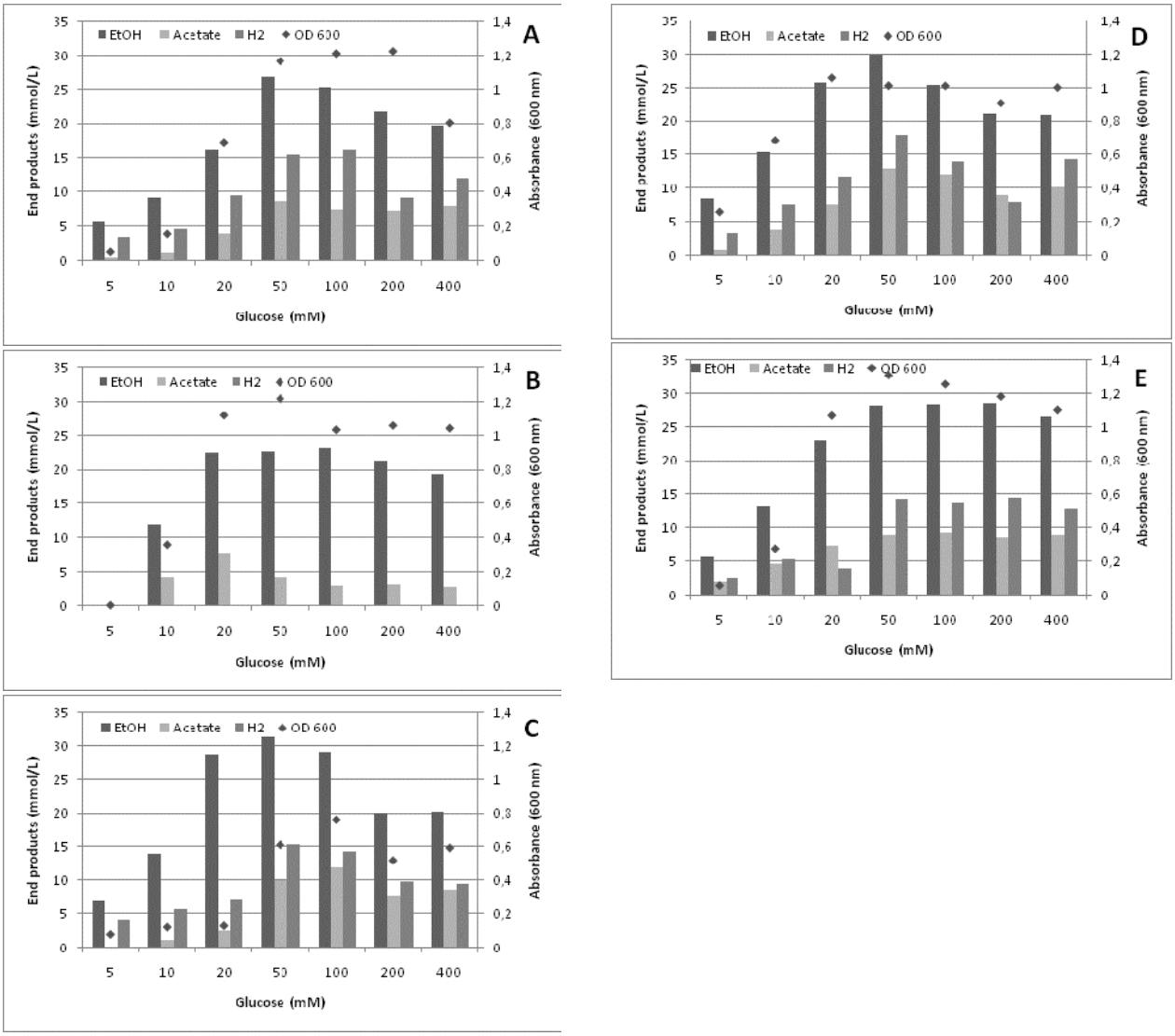


Figure 4.4. End product formation for five thermophilic bacteria at different initial glucose loadings. The strains are 15-06-G (A), 25-07-C (B), 64-07-X (C), 66-07-P (D) and 67-07-P (E).

One of the main issues of using a thermophilic bacteria for bioethanol production from complex biomass is, apart from producing high yields of ethanol, their tolerance towards high initial substrate concentration. It is well known that fermenting microorganisms can have limited tolerance towards increased substrate loadings. Therefore, different initial glucose loadings were used for the five isolates, ranging from

5 mM to 400 mM (Fig. 4.4.A-E). The fermentation spectrum was similar for all strains: at low glucose loading, usually up to 20 mM or 50 mM a good correlation was in the increase of end product formation. Additionally, all glucose was degraded in the culture broth. However, at 50 mM glucose concentrations, only a slight increase was in the formation of end products and substantial amounts of glucose were left in the fermentation broth. This inhibition was not at the same substrate concentrations for all strains. Strain 15-06-G was not clearly inhibited until at 100 mM glucose concentration but the other on 50 mM concentrations.

End product formation from various substrates. The five strains were cultivated on more than 30 carbon substrates (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, threonine, histidine and cysteine). Figure 4.5. shows end product formation from substrates that were positive and resulted in biomass formation. All substrates were used in 20 mM concentrations and in the case of pectin and xylan, 3 g L^{-1}. In general the strains were highly saccharolytic, degrading most of the pentoses, hexoses and disaccharides tested. Additionally, all strains utilized pectin and xylan but end product formation varied between the strains. Finally, some of the strains grew on pyruvate and the amino acid serine.

Strain 15-06-G degraded all carbohydrates tested except for the C5-sugar arabinose (Fig. 4.5.A). End product formation was similar for all the hexoses and the stochiometry for glucose fermentation is:

1.0 Glucose → 0.9 EtOH + 0.5 Acetate + 1.4 CO_2 + 0.7 H_2

Most ethanol was produced on lactose but the strain could also produce substantial amounts of ethanol from the polymeric substrates pectin and xylan. Finally, pyruvate was clearly decarboxylated to acetyl-CoA and acetate was the sole volatile end product.

Strain 25-07-C was the only strain isolated on cellulose and is phylogenetically distanced away from the other four isolates (Fig. 4.1.). The strain produces ethanol and

acetate as main end products but no hydrogen production occurred on any of the substrates tested. The only carbohydrate not utilized was arabinose. Similar values of ethanol and acetate were produced on most substrates with the ratio approximately 20 mM of ethanol and 12 mM of acetate from 20 mM of substrate.

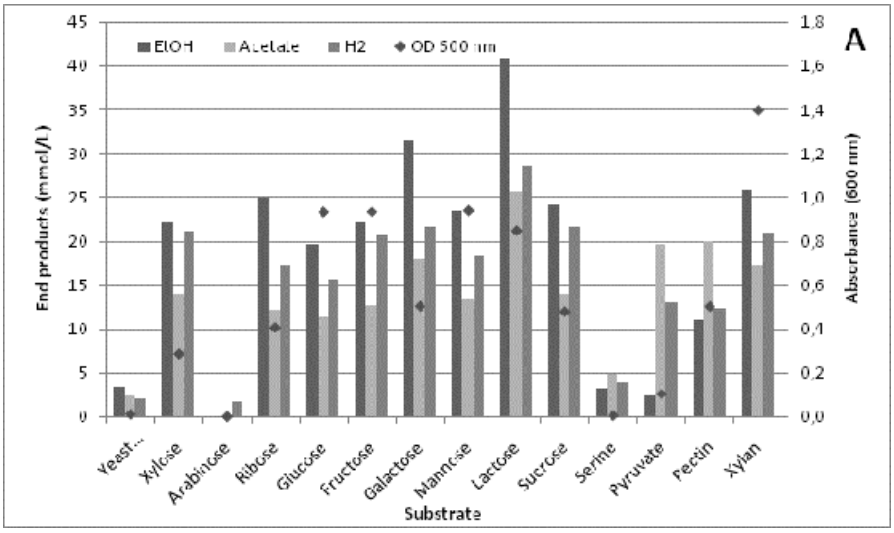
Strain 64-07-X was isolated on xylan and degraded, as in the case of 15-06-G and 25-07-C, all carbohydrates except for arabinose. The strain produced approximately 1 mol-EtOH/mol-pentose and between 0.6 to 1.6 mol-EtOH/mol hexose degraded (assuming all sugars were completely degraded). Highest ethanol concentrations observed were on lactose (35 mM). The other volatile end product analyzed in the culture broth was acetate but in less amounts. The stochiometry on glucose fermentation for this strain is:

1.0 Glucose → 1.4 EtOH + 0.6 Acetate + 2.0 CO_2 + 1.1 H_2

The strain could grow on pyruvate but with little biomass and end product formation (solely acetate).

Strain 66-07-P was isolated on pectin and showed similar fermentation spectrum as for the other strains and could for instance not degrade arabinose. Similar amounts of ethanol and acetate were produced from most of the monosugars, 18 to 22 mM and 10 to 24 mM, respectively. Higher end product formation was however observed on the disaccaharides. This strain did not grow on serine and pyruvate but grew well on pectin and xylan as the other strains.

Strain 67-07-P was the only strain that grew on all simple carbohydrates tested, including arabinose. Concentration of end products from most of the monosugars were between 18 to 20 mM of ethanol, 7 to 10 mM of acetate and 2 to 20 mmol L^{-1} of hydrogen. An exception from this spectrum was observed on galactose where more than 30 mM of ethanol were produced which is in similar range as from the disaccharide lactose and sucrose. The strain produced mainly acetate and hydrogen from pyruvate.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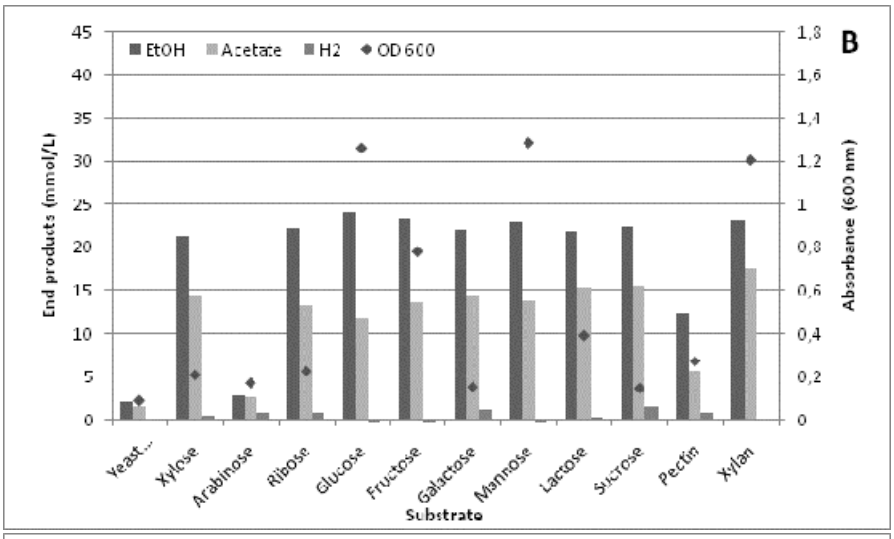
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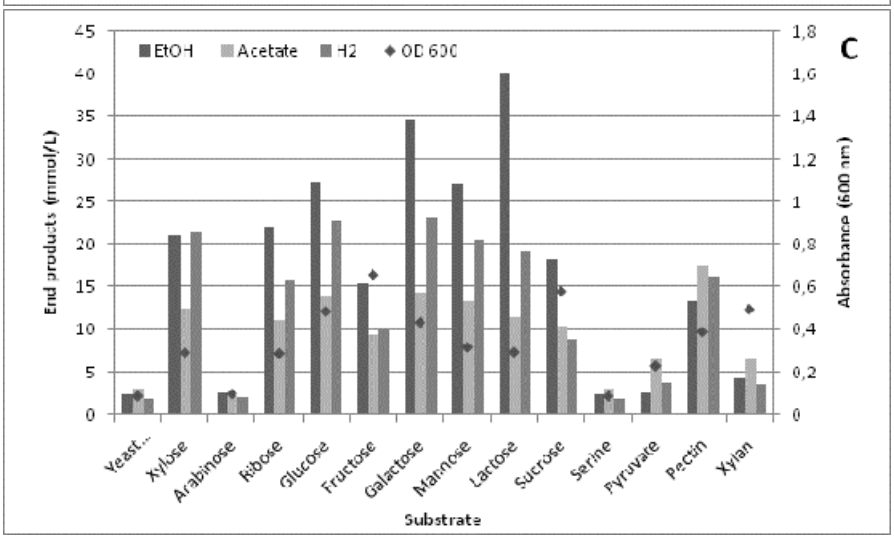
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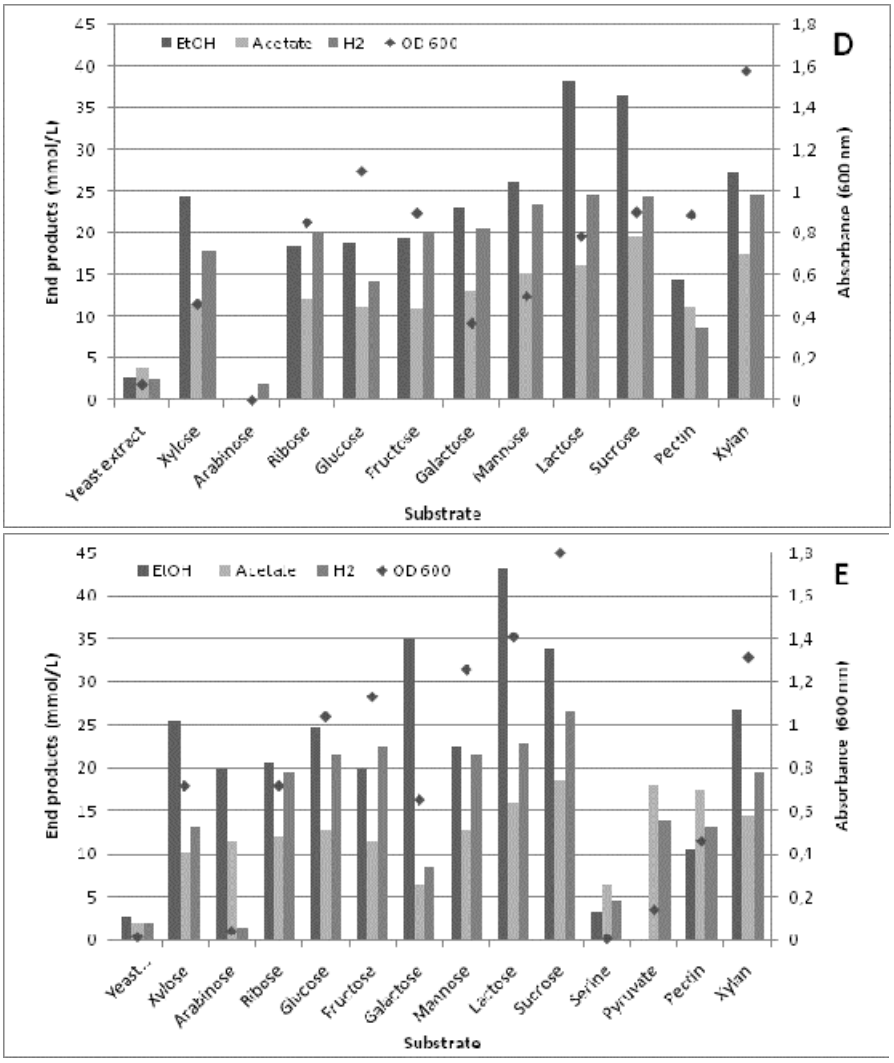

Figure 4.5. End product formation for five thermophilic bacteria from various carbon substrates. The strains are 15-06-G (A), 25-07-C (B), 64-07-X (C), 66-07-P (D) and 67-07-P (E). Initial substrate concentration was 20 mM in all cases except for pectin and xylan (3 g L^{-1}).

5. Manuscript I

Bioprospecting of ethanol and hydrogen producing bacteria from Icelandic hot springs.

Bioprospecting of ethanol and hydrogen producing bacteria from Icelandic hot springs

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AND

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Abstract

A total of 68 samples were collected from hot springs varying in temperatures from 40 – 93°C and in pH's of 2.5 – 9.6. The samples were enriched on various carbohydrate polymers (cellulose, xylan, pectin (3 g L^{-1} in all cases) and monosugars (glucose and xylose (50 mM in both cases) under anaerobic conditions at four different temperatures (50, 60, 70 and 75°C) and at two pH values (6.0 and 7.0). Positive enrichments were re-inoculated into the same carbon source several times and from the final enrichments, end point dilutions were made and samples submitted for partial 16S rRNA analysis. A total of 60 samples were analyzed and 49 gave good sequences which were used for phylogenetic analysis. Most of the lower 50°C - enrichments indicated the presence of bacteria belonging to Clostridia Cluster V (*Thermoanaerobacterium* mostly) and Cluster I (*Caloramator* and *Clostridium*). At higher temperatures the *Thermoanaerobacterium* and *Thermoanaerobacter* were more dominant and at 70 and 75°C only members of the genera *Thermoanaerobacter* and *Caldicellulosiruptor* were present. End product formation from 50 mM of glucose and xylose were performed for all enrichments. Low temperature enrichments (50°C) produced mainly ethanol, acetate and butyrate from monosugar fermentation and two enrichments produced more than 1.3 mol-EtOH/mol-glucose and two enrichments produced more than 1.0 mol-EtOH/mol xylose. Most of the enrichments that directed their carbon flow to ethanol belong to *Caloramator*, *Paenibacillus* and *Thermoanaerobacterium*. At higher temperatures, several enrichments produced more than 1. 0 mol-EtOH/mol-glucose and xylose, especially at 60°C, mostly belonging to *Thermoanaerobacter* and *Thermoanaerobacterium*. Butyrate was only detected in one of the 18 enrichments at 60°C and not at higher temperatures. Only one enrichment of a total of 16 at 70 and 75°C produced more than 1.0 mol-EtOH/mol glucose but the carbon flow was directed more towards acetate and hydrogen formation.

Key words: thermophilic bacteria, hydrogen, ethanol,

1. Introduction

Today, the world faces a progressive depletion of its energy sources, mainly based on non-renewable fuels. The use of fossil fuels has also resulted in resource depletion, environmental deterioration and public health problems. Therefore, a demand to develop novel renewable energy harvesting technologies and to introduce sustainable energy carriers exists (Zaldivar, Nielsen, & Olsson, 2001) (Sánchez & Cardona, 2008). One renewable solution is the use of solar energy represented in energy crops and lignocellulosic residues. Conversion of this biomass into biofuels is very important for the exploitation of alternative energy sources and the reduction of greenhouse gases (Hamelinck, van Hooijdonk, & Faaij, 2005). Ethanol from biomass (bioethanol) is the most employed liquid biofuel either as a fuel or as a gasoline enhancer. Hydrogen from biomass as a potential biofuel has also been addressed lately by the use of fermentative bacteria (Koskinen, et al., 2008a) (Koskinen, et al., 2008b) (Kumar & Das, 2001). Both hydrogen and ethanol can be obtained from energy crops and lignocellulosic material. The complexity of the production processes depends on the feedstock. Thus, implemented technologies span from simple conversion of sugar and starch based biomass to multi-stage conversion of lignocellulosic material (Sánchez & Cardona, 2008). The main feedstock for ethanol production is sugar cane in Brazil and from starch based biomass in US (American Coalition for Ethanol, 2009) (Sánchez & Cardona, 2008). In both cases monosugars are degraded with high yields to ethanol by yeast fermentation. However, lignocellulosic material is the most abundant biopolymer on Earth and its annual production is estimated in approximately 50 billion ton (Claassen, et al., 1999). Many types of lignocellulosic material have been tested for bioethanol production varying from crop residues to hardwood, waste paper and municipal solid waste (Zaldivar, Nielsen, & Olsson, 2001). Various pre-treatment methods have been developed for the utilization of lignocellulosic material prior to fermentation (Sánchez & Cardona, 2008). One of the main problems in bioethanol production from lignocellulosic material is that most yeast species cannot ferment the pentose containing sugars (e.g. xylose and arabinose) which often comprise a substantial portion of the biomass. Many investigations to overcome this obstacle have been performed through genetic engineering of the microbes involved (Dien, Cotta, & Jeffries, 2003). Additionally, pentose fermenting yeasts have also been used like *Pichia stipitis*, *Candida shehatae* and *Pachysolen tannophilus* but their ethanol production

rates are at least five times slower as compared to *Saccharomyces cerevisae* (Agbogbo & Coward-Kelly, 2008) (Sreenath, et al., 2001) (Bravo, et al., 1995) (Ingledew, 1999)
Hydrogen production through fermentation has received increasing attention in the past few years. High H_2 production rates, up to 15 l h^{-1} l^{-1} of reactor volume (i.e 360 l d^{-1} l^{-1}) (Wu, et al., 2006) have been achieved with mesophilic H_2 fermentation processes, and a long term stability of the H_2 fermentation processes has been demonstrated (Lin & Chang, 2004) (Yu & Mu, 2006). Thermophilic H_2 fermentation has many advantages to mesophilic fermentation, but has remained less studied. High temperatures favour the stoichiometry of H_2 production resulting in higher H_2 yields compared to mesophilic systems (van Groenestjin, et al., 2002) (van Niel, Claassen, & Stams, 2003). Furthermore, thermophilic fermentation results in less variety of end products as compared to those of mesophilic fermentation (van Niel, Claassen, & Stams, 2003). The highest hydrogen yields in pure cultures have been shown by *Caldicellulosiruptor saccharolyticum* (de Vrije, et al., 2007)and *Thermotoga elfii* (van Niel, et al., 2002). Recently, enrichment culture from the geothermal area in Hveravellir in Iceland was reported with very high hydrogen yields (3.2 mol H_2/mol-glucose) (Koskinen, et al., 2008b)

Thermophilic bacteria have recently been considered for fermentation of lignocellulosic material. The most important groups of ethanol and/or hydrogen producing thermoanaerobes are the thermophilic clostridia and members within the genera *Thermoanaerobacter*, *Thermoanaerobacterium, Caldicellulosiruptor and Thermotoga.* Many species within these genera can produce relatively high amounts of ethanol and hydrogen from pentoses and hexoses. (Wiegel & Ljungdahl, 1981; Kublanov, et al., 2007) (de Vrije, et al., 2007) (van Niel, et al., 2002). The main drawback of using these microbes is the production of by-products (acetate, lactate, butyrate and formate) and their very low ethanol tolerance (Wiegel, 1980). However, the fact that the culture may carry out their ethanol production at high temperatures offers the possibility to remove the alcohol by distillation or evaporation (Claassen, et al., 1999)
Geothermal springs have been regarded as potential source for ethanol and hydrogen producing bacteria (Koskinen, et al., 2008a) (Sommer, Georgieva, & Ahring, 2004) (Wiegel & Ljungdahl, 1981). Present investigation is towards the bioprospecting of various ethanol and hydrogen producing bacteria isolated from more than 40 hot springs

varying in temperatures and pH's. All samples were subjected to partial 16S rRNA analysis and tested for their ethanol and hydrogen production capacity in batch assays.

2. Materials and methods

2.1. Sampling sites

Sixty eight samples were collected in June 2007 from the Hengill-area in Graendalur in SW of Iceland. The temperature of the hot springs ranged from 40.0°C to 93.4°C and the pH was between 2.5 and 9.6. Forty four of the samples were used for enrichment of ethanol and hydrogen producing bacteria based on the pH and temperature of hot springs. Additionally, several samples collected from the same area in 2006 are included in this study.

2.2. Medium

The medium (per liter) consisted of: NaH_2PO_4 0.58 g, Na_2HPO_4 1.36 g, 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, resazurine 1 mg, trace element solution 1 ml, vitamin solution 1 ml and $NaHCO_3$ 0.8 g. Carbon and energy sources were 20 mM or in the case of polymers, 3 g l^{-1}. The vitamin solution was according to DSM141. The trace element was as described earlier (Orlygsson and Baldursson, 2007). The medium was prepared by adding the buffer to distilled water, which was then boiled for 5-10 min and cooled while flushing with nitrogen. The mixture was then transferred to cultivation bottles using the Hungate technique (Hungate 1969) and then autoclaved. All other components of the medium were added separately through filter sterilized solutions.

2.3. Isolation and Enrichments

Samples were collected using an extended pole equipped with grip arms placed at the end of it. Serum bottles (120 ml) were fixed at the end, opened and completely filled with geothermal liquid/mud samples and closed with butyl rubber and aluminum caps. The temperature was measured after sampling directly into the hot springs. Upon arrival to the laboratory, the pH was measured. Samples in the pH-range 5.5 - 7.5 were selected for enrichments. A five ml aliquot from each sample was inoculated into120 ml serum bottles containing 45 ml medium with 2 g l^{-1} yeast extract and either 50 mM monosugars (glucose or xylose) or 3 g l^{-1} of xylan, pectin or cellulose (both Whatman

paper and cellulose powder). The samples were incubated at temperatures slightly below the experimental site temperatures (50°C, 60°C, 70°C and 75°C) and at pH 6.0 and 7.0. Samples were incubated for one week and positive enrichments (determined by overpressure measured by a syringe or hydrogen production (GC)) were re-inoculated to the same media. This was repeated three times (positive samples only). Positive samples from the third enrichment series were diluted with end point dilutions (two times) and sent for partial 16S rRNA analysis.

2.4. 16 S rRNA sequence anlysis

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

2.5. End product formation from carbohydrates

End product formation from glucose and xylose was done by inoculating 1 ml of a fresh culture into 49 ml of medium containing the carbon substrate. The concentration of substrates was 50 mM or in the case of polymers 3 gL^{-1}. Fermentation time was one week and samples for volatile fatty acids, ethanol and hydrogen were taken and analyzed at the beginning and in the end of the experimental time.

2.6. Analytical methods

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

3. Results

3.1. Temperature and pH's of sampling sites – Isolation procedure

A total of 68 samples were collected from hot springs in Graendalur close to Hveragerdi in SW-Iceland. The hot springs had temperatures varying from 40.0 to 93.4°C and pH's varying from 2.5 to 9.6. The pH was used as selection criteria and samples with pH between 4.9 and 9.6 were further used for enrichments. Thus, forty four samples were inoculated on the six different carbon sources (264 enrichments). After several repeated enrichments on the various carbon substrates the number of samples had reduced to 58 (Table 1). Additionally two samples already obtained in earlier studies in our group were included for further treatment of the samples. These samples were analyzed for phylogenetic characterization by 16S rRNA analysis. Most of the enrichments (82%) gave good sequences (Tables 2 – 5).

Table 1. The numbering and names of samples, temperature and pH of the hot spring samples were collected from and the temperature and pH media the samples were incubated at. Labelling of the samples are: G = enriched on glucose, Xo = xylose. Cpa = cellulose (Whatman paper), Cpo = cellulose (powder), Cpa-G = cellulose at start of enrichment but later on enriched on glucose, P = pectin, X= xylan. Number of hot springs are indicated and year of isolation.

Sampling site	Culture	Temperature (site - incubation)	pH (site - incubation)	Sampling site	Culture	Temperature (site - incubation)	pH (site - incubation)
1	1-07-Cpa-G	45/50°C	8.0/7.0	27	27-07-X	60/60°C	7.7/7.0
2	2-07-G	50/50°C	7.9/7.0	29	29-07-G	60/60°C	9.6/7.0
2	2-07-Cpa-G	50/50°C	7.9/7.0	29	29-07-Cpo	60/60°C	9.6/7.0
2	2-07-Cpa	50/50°C	7.9/7.0	29	29-07-Cpa-G	60/60°C	9.6/7.0
9	9-07-P	40/50°C	4.8/6.0	34	34-07-X	60/60°C	7.4/7.0
9	9-07-X	40/50°C	4.8/6.0	54	54-07-Xo	66/60°C	5.3/6.0
10	10-07-P	46/50°C	6.6/7.0	54	54-07-P	66/60°C	5.3/6.0
10	10-07-X	46/50°C	6.6/7.0	63	63-07-G	60/60°C	7.7/7.0
15	15-06-G	49/50°C	5.8/7.0	63	63-07-Cpa	60/60°C	7.7/7.0
15	15-07-Cpa-G	50/50°C	7.5/7.0	64	64-07-G	59/60°C	7.0/7.0
23	23-07-Cpa-G	57/50°C	7.7/7.0	64	64-07-P	59/60°C	7.0/7.0
25	25-07-Cpa-G	50/50°C	7.4/7.0	64	64-07-X	59/60°C	7.0/7.0
35	35-07-X	50/50°C	7.7/7.0	66	66-07-G	62/60°C	7.4/7.0
35	35-07-Cpa-G	50/50°C	7.7/7.0	66	66-07-P	62/60°C	7.4/7.0
44	44-07-G	56/50°C	5.5/6.0	67	67-07-P	66/60°C	7.7/7.0
44	44-07-Xo	56/50°C	5.5/6.0	2	2-06-Cpa-G	73/70°C	4.3/7.0
44	44-07-P	56/50°C	5.5/6.0	4	4-07-X	69/70°C	8.0/7.0
44	44-07-X	56/50°C	5.5/6.0	8	8-07-Cpo	71/70°C	8.2/7.0
47	47-07-Xo	53/50°C	6.2/6.0	8	8-07-Cpa	71/70°C	8.2/7.0
47	47-07-P	53/50°C	6.2/6.0	20	20-07-G	69/70°C	7.5/7.0
52	52-07-Xo	50/50°C	5.4/6.0	20	20-07-Cpo	69/70°C	7.5/7.0
52	52-07-P	50/50°C	5.4/6.0	33	33-07-Xo	71/70°C	8.0/7.0
65	65-07-Xo	49/50°C	6.7/7.0	55	55-07-Xo	73/70°C	5.1/6.0
65	65-07-X	49/50°C	6.7/7.0	14	14-07-G	84/75°C	8.0/7.0
8	8-06-G	58/60°C	6.2/6.0	14	14-07-X	84/75°C	8.0/7.0
21	21-07-Xo	60/60°C	6.7/7.0	14	14-07-P	84/75°C	8.0/7.0
21	21-07-Cpo	60/60°C	6.7/7.0	14	14-07-X	84/75°C	8.0/7.0
21	21-07-Cpa-G	60/60°C	6.7/7.0	32	32-07-G	78/75°C	4.9/6.0
24	24-07-X	60/60°C	7.7/7.0	39	39-06-G	78/75°C	5.5/6.0

3.2. 16S rRNA gene sequence analysis

Samples that were analyzed for 16S rRNA analysis revealed that in most cases pure cultures had been obtained, although several samples, especially those enriched on cellulose, gave bad sequence data. In some of these cases, samples were re-enriched on glucose and then submitted for 16S rRNA analysis. Thus, of the total of 60 samples that were analysed for partial 16S rRNA analysis 49 samples revealed good sequences. The data obtained was used to determine the phylogenetic position of the bacteria and are presented for each of the four enrichment temperature used (Fig. 1 – 4).

3.2.1. 16S rRNA gene sequence analysis from 50°C-enrichments

A total of 20 samples out of 24 were sequenced for partial 16S rRNA analysis from enrichment cultures at 50°C (Figure 1; Table 2). Three samples gave bad sequences and are not shown. Most bacteria (15 out of 17 samples) were classified within various clusters of *Clostridium* and two major sub-clusters were observed; one containing species within the cluster V (*Thermoanaerobacter* and *Thermoanaerobacterium*) (9 samples) and another with species within cluster I (6 samples; Fig. 1) belonging to genera of *Caloramator* and *Clostridium*. Samples enriched directly on monosugars, xylan and pectin mostly rendered bacteria that belong to *Thermoanaerobacter* and *Thermoanaerobacterium* except for sample 15-06-G (*Caloramator*) and 9-07-X which belongs to the Cluster I. Most of the *Thermoanaerobacterium* enrichments showed close relationship to each other and to species like *T. aciditolerans, T. saccharolyticum, T. islandicum, T. aotearoense* and *Thermoanaerobium lactoethylicum*. For instance, strains 44-07-P and 47-07-P were identical and had 99.7% homology with strain 52-07-Xo. These three isolates were all closely related with *T. aotearoense* (99.4 to 99.7% homology) and *T. saccharolyticum* (98.8 to 99.1% homology). Strains 9-07-P and 35-07-X were also closely related to each other (99.7%) and to *T. saccharolyticum* (99.1 to 99.7%) and *T. aciditolerans* (98.8 to 99.1%). In general, phylogenetic relationship between the eight enrichments that belong to *Thermoanaerobacterium* ranged from 97.7% to 100% with the five *Thermoanaerobacterium* species showed in figure 1. Enrichment 44-07-G was the only sample that originated from 50°C and showed close homology (99.7%) to *Thermoanaerobacter thermohydrosulfuricus* and *Thermoanaerobacter wiegelii*. In many cases, sequence analysis of samples that originated from cellulose enrichments gave bad sequences. These samples were therefore inoculated and enriched on glucose and analyzed phylogenetically as well (a total of six samples; labeled with Cpa in Fig. 1).

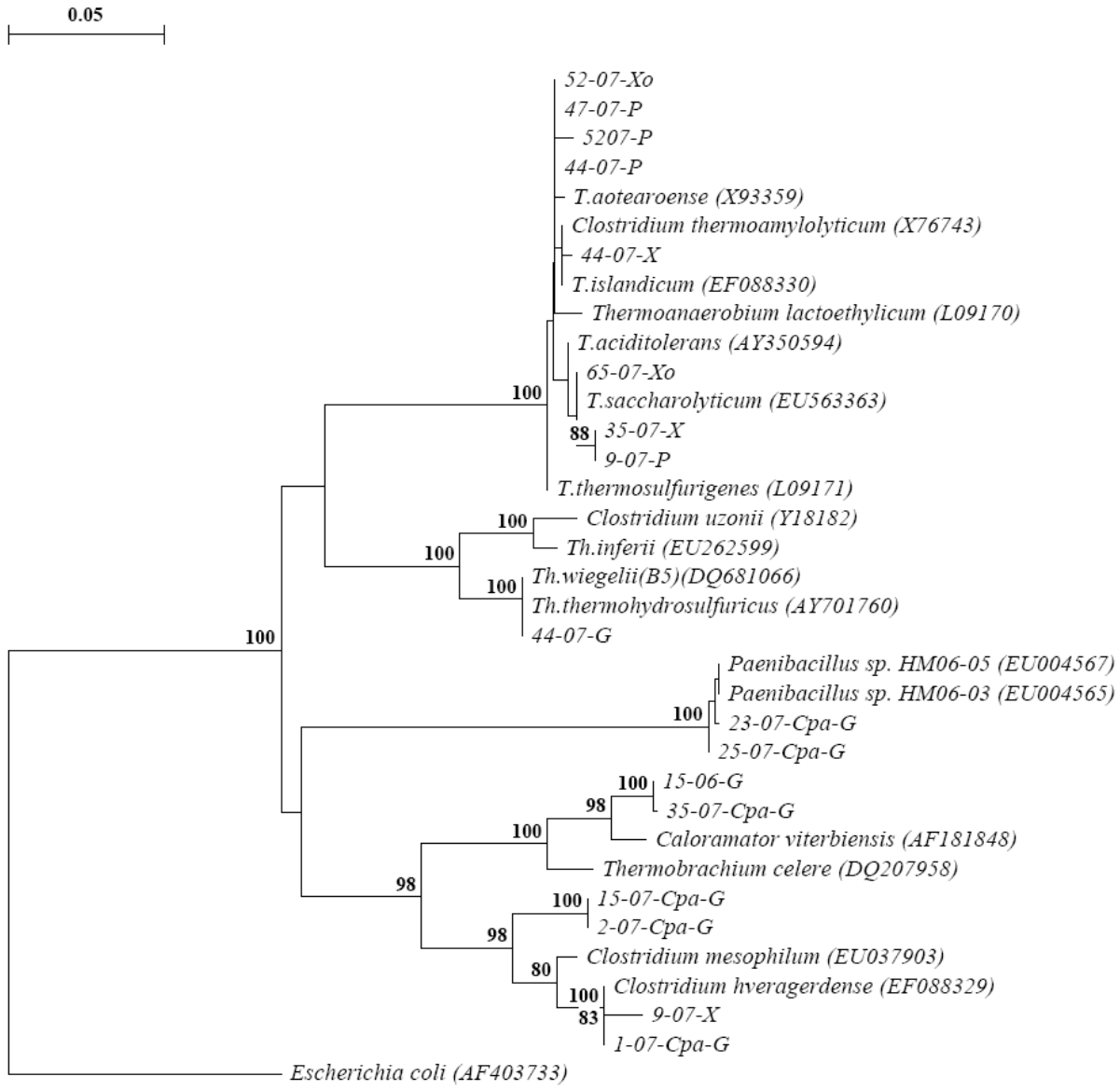

Figure 1. Phylogeny of the strains based on the 16S rRNA gene partial sequences (≈500 bp) from enrichments at 50°C. The phylogenetic tree was generated using a distance matrix and neighbor joining algorithms with 500 bootstraps. Only supported bootstrap values (>80%) are shown. Escherichia coli (AF403733) was selected as an out-group. The scale bar indicates 0.05 substitutions per nucleotide position.

Four of these samples showed bacterial relationship with *Clostridium* cluster I and two to the genus *Paenibacillus*. Enrichments 35-07-Cpa-G and 15-06-G showed 99.7% similarity to each other and were closely related to 98.3% homology to *Caloramator viterbensis*. Analysis of four enrichments resulted in species with close relationship to

Clostridium hveragerdense and *Clostridium mesophilum* strain SW408. Enrichments 2-07-Cpa-G and 15-07-Cpa-G were closely related (99.7% homology) and had 96.1 to 96.3% homology to *C. hveragerdense*, a strain that was isolated from the same geothermal area as these samples (Örlygsson and Baldursson, 2007). Enrichments 1-07-Cpa-G and 9-07-X also belong to *Clostridium* cluster I and had 97.5% homology to each other and between 97.5 to 99.1% to *C. hveragerdense*.

3.2.2. *16S rRNA gene sequence analysis from 60°C-enrichments*

Eighteen of the 20 samples that were sequenced for partial16S rRNA analysis from 60°C revealed good sequences indicating that pure cultures had already been obtained (Fig. 2; Table 3). Enrichments from hot spring number 54 isolated on pectin and xylose were identical and most closely related to *Thermoanaerobacterium aotearoense*, *T. islandicum* and *Clostridium thermoamylolyticum* (99.7%). Strains 66-07-G and 67-07-P were also very close to these bacteria (99.4%) and were identical to each other. Six enrichments (24-07-X, 34-07-X, 8-06-G, 66-07-P, 21-07-Xo and 64-07-P) were identical and very close to the four above mentioned enrichments as well as to *Thermoanerobacter saccharolyticum* and *T. aciditolerans* (> 98.0% homology). The only enrichment classified within the genus *Thermoanaerobacterium* that showed low homology was 64-07-X (89.1% homology to *Clostridium thermoamylolyticum*) but the sequence data for this sample was of low quality. Four enrichments were classified within the genus *Thermoanerobacter*. Enrichment 63-07-G, 29-07-Cpa-G and 29-07-G were identical and very close to *Thermoanaerobacter wiegelii, Th. thermohydrosulfuricus* (100% homology) and *Th. ethanolicus* (99.7% homology).

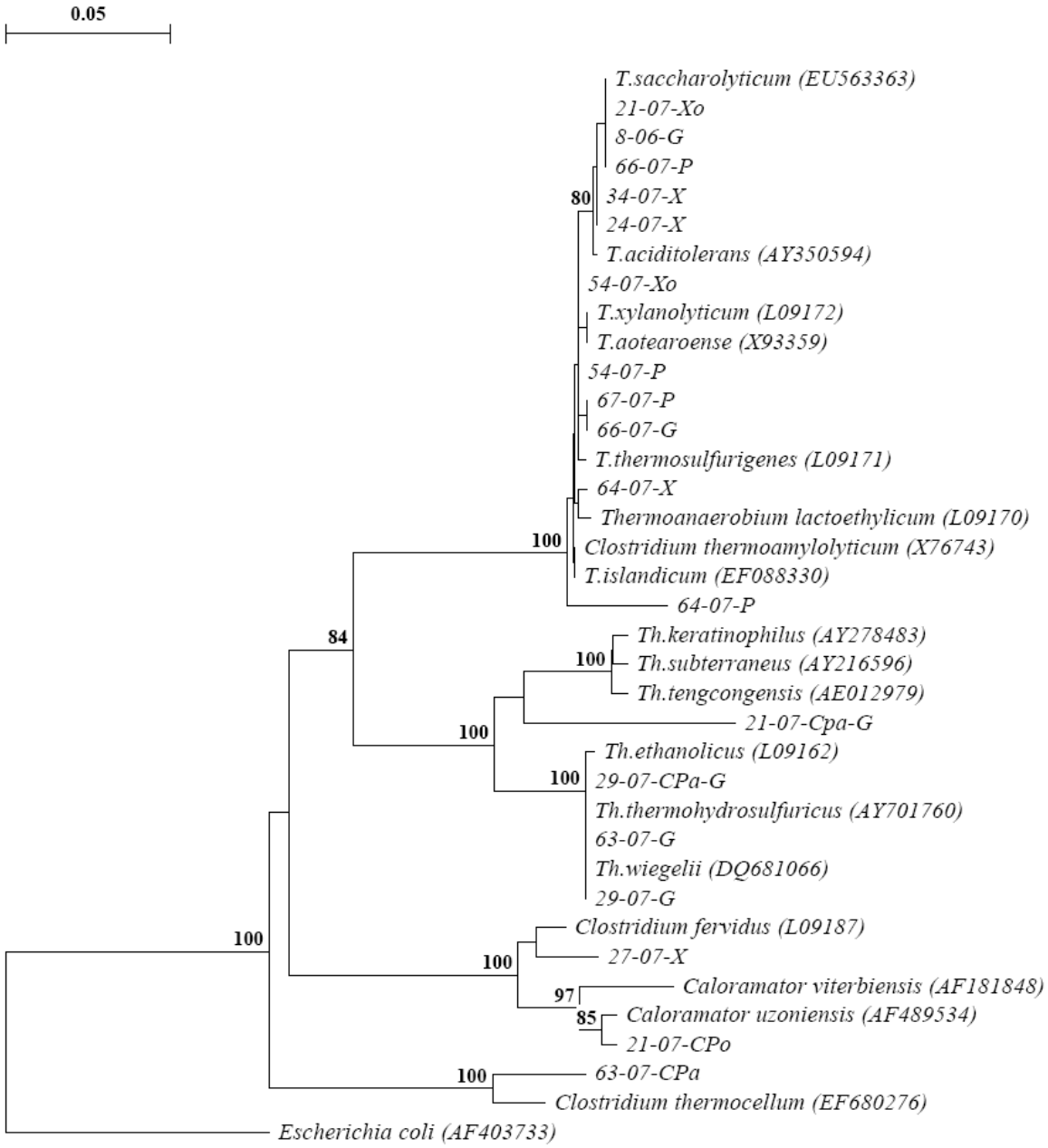

Figure 2. Phylogeny of the strains based on the 16S rRNA gene partial sequences (≈500 bp) from enrichments at 60°C. The phylogenetic tree was generated using a distance matrix and neighbor joining algorithms with 500 bootstraps. Only supported bootstrap values (>80%) are shown. Escherichia coli (AF403733) was selected as an out-group. The scale bar indicates 0.05 substitutions per nucleotide position.

Three enrichments were the classified within the family Clostridiaceae. Enrichment 63-07-Cpa belongs to the genus *Clostridium* with *C. thermocellum* as the closest relative (95.2%). Enrichments 21-07-Cpa and 27-07-X belong to *Caloramator* with *Caloramator uzoniensis* and *Clostridium fervidus* as closest relatives (99.2 and 97,4% homology).

3.2.3. *16S rRNA gene sequence analysis from 70°C-enrichments*

Ten enrichment cultures at 70°C were analysed for 16S rRNA and nine of them gave good data for phylogenetic analysis. The four cultures originating from glucose and xylose all belong to the genus *Thermoanaerobacter* whereas the four strains analyzed on polymers belong to either *Thermoanaerobacter* or *Caldicellulosiruptor* species (Figure 3; Table 4). The 16S rRNA sequence analysis of enrichments 33-07-Xo and 55-07-Xo were identical and these strains were very close (98.0 – 100%) to well known species within *Thermoanaerobacter* species (*T. thermocopriae, T. thermohydrosulfuricum, T. wiegelii, T. ethanolicus* and *T. sideriophilus*). Enrichment 20-07-X was also close to these bacteria and had 98.0% homology with 33-07-Xo and 55-07-Xo. Enrichment 20-07-G however belongs to another sub-cluster of *Thermoanaerobacter* with 98.0 and 99.0% homology to *T. tengcongensis* and *T. keratinophilus*, respectively. Two other enrichments were classified within this genus, i.e. 2-06-Cpo-G and 20-07-Cpo, both isolated on cellulose powder. These enrichments showed 98.0% homology and were most closely related to *Thermoanaerobacter inferii, T. uzonii* and *Clostridium uzonii* (95.0 to 97.0% homology). Finally, three strains were identified to belong to *Caldicellulosiruptor*.

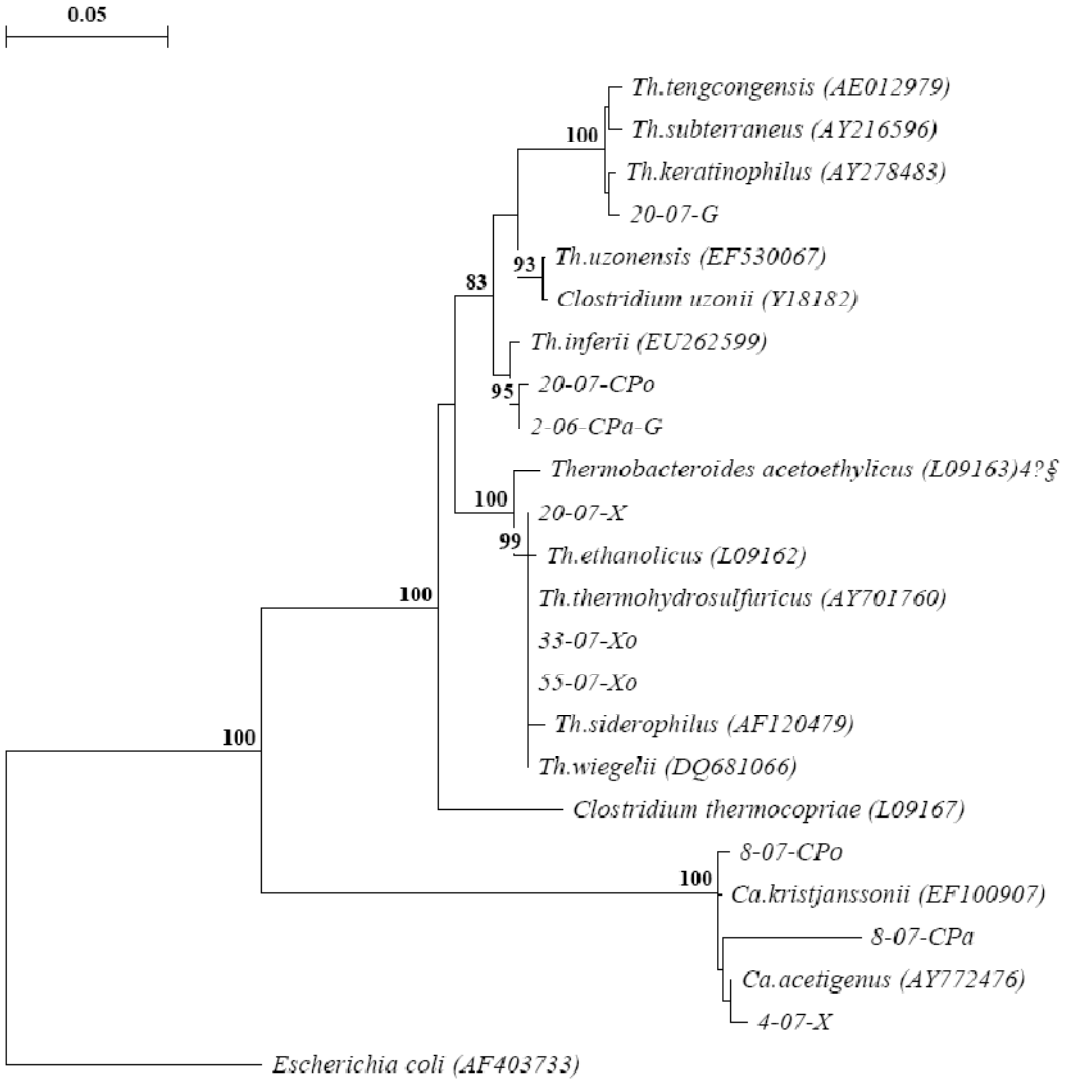

Figure 3. Phylogeny of the strains based on the 16S rRNA gene partial sequences (≈500 bp) from enrichments at 70°C. The phylogenetic tree was generated using a distance matrix and neighbor joining algorithms with 500 bootstraps. Only supported bootstrap values (>80%) are shown. Escherichia coli (AF403733) was selected as an out-group. The scale bar indicates 0.05 substitutions per nucleotide position.

3.2.4. *16S rRNA gene sequence analysis from 75°C-enrichments*

From six 16S rRNA sequence analysis of the highest temperature enrichment samples, only three could be used for phylogenetic studies. These three samples all revealed bacteria that belong to the genus *Thermoanaerobacter* (Figure 4). The two enrichments

isolated on polymeric carbohydrates, 14-07-X and 39-06-P, showed 95.3% homology to each other. Strain 14-07-X was phylogeneticially very close to *Thermoanaerobacter keratinophilus* and *T. subterraneus* (99.0 and 99.2%), respectively, but strain 39-06-P was distanced further away from these two species (94.7 and 94.9%). Enrichment 14-07-Xo was however analyzed as *Thermoanaerobacter wiegelii* (100% homology).

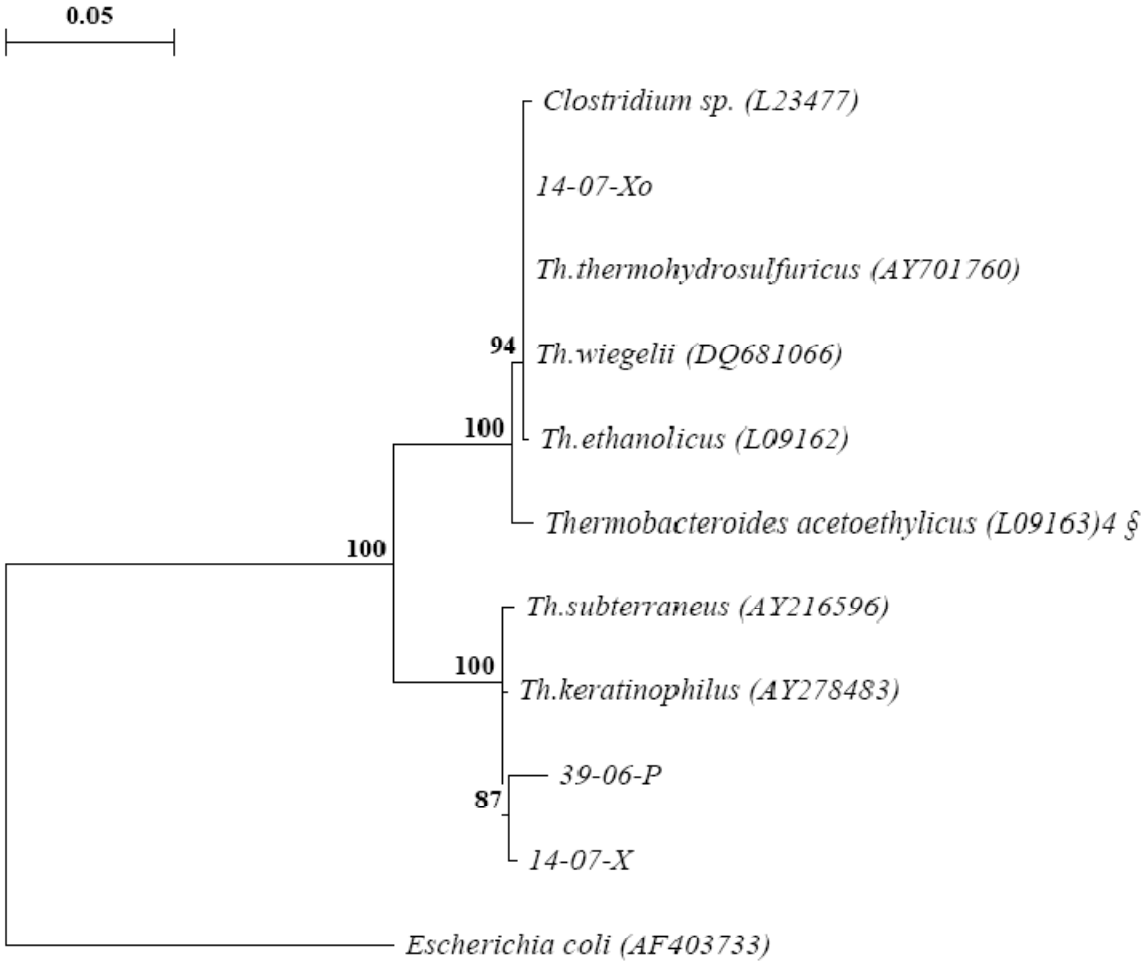

Figure 4. Phylogeny of the strains based on the 16S rRNA gene partial sequences (≈500 bp) from enrichments at 75°C. The phylogenetic tree was generated using a distance matrix and neighbor joining algorithms with 500 bootstraps. Only supported bootstrap values (>80%) are shown. Escherichia coli (AF403733) was selected as an out-group. The scale bar indicates 0.05 substitutions per nucleotide position.

3.3. End product formation from enrichments

3.3.1. End product formation from 50°C-enrichments

Two enrichment cultures (15-06-G and 25-07-Cpa) that produced the highest amounts of ethanol from glucose belong to *Caloramator* and *Paenibacillus* (Tables 2 and 6), both with more than 1.3 mol-ethanol/mol-glucose. The best ethanol producers on xylose were enrichments 44-07-G and 65-07-Xo (> 1.0 mol-ethanol/mol-xylose) belonging to *Thermoanaerobacter* and *Thermoanaerobacterium*, respectively. Other "good ethanol producers" on glucose (> 0.7 mol-EtOH/mol-glucose) were 44-07-Xo (16S rRNA analysis not done) and 35-07-Cpa (*Caloramator viterbensis*; 94%) and on xylose (> 0.6 mol EtOH/mol xylose) were 2-07-G, 15-06-G, 44-07-Xo, 2-07-Cpo, 47-07-P, 52-07-P and 65-07-P.

Table 2. The main phylogenetic and physiological data of enrichment cultures from 50°C. End product formation from 50 mM of glucose and xylose in mM concentrations are shown as well as the closest phylogenetic position of the enrichment in percentages.

Sample	Ethanol (mM)		Acetate (mM)		Butyrate (mM)		H2 (mmol L-1)		Phylogeny
Substrates	Glucose	Xylose	Glucose	Xylose	Glucose	Xylose	Glucose	Xylose	
2-07-G	21	32	21	21	0	0	30	27	Not analysed
15-06-G	66	31	16	17	0	0	22	20	*Caloramator viberbiensis* (93,5%)
44-07-G	28	51	16	15	0	0	21	23	*Thermoanaerobacter* species (99%)
44-07-Xo	35	39	21	23	0	0	24	29	Not analysed
47-07-Xo	25	16	16	10	0	0	22	18	Not analysed
52-07-Xo	28	27	16	15	0	0	20	27	*Thermoanaerobacterium* sp (98%)
65-07-Xo	7	57	8	12	12	0	20	14	*Thermoanaerobacterium* sp (98 - 99%)
1-07-Cpa	8	14	6	9	2	1	0	0	*Clostridium hveragerdense* strain AK14 (99%)
2-07-Cpa	9	21	6	14	1	1	6	3	*Clostridium* sp. strain P2 (94%)
15-07-Cpa	8	4	19	4	1	8	9	1	*Clostridium* sp. strain P2 (94%)
23-07-Cpa	12	24	10	13	1	0	4	5	*Paenibacillus* sp. HM06-04 (99%)
25-07-CPa	67	17	15	14	1	1	17	15	*Paenibacillus* sp. HM06-04 (99%)
35-07-Cpa	47	6	17	6	1	1	19	8	*Caloramator viberbiensis* (94%)
2-07-Cpo	40	31	15	18	1	0	8	12	Not analysed
9-07-P	19	22	14	17	0	0	20	23	*Thermoanaerobacterium* sp. (97-98%)
10-07-P	2	21	11	18	14	1	24	15	Not analysed
44-07-P	25	21	21	14	6	0	31	23	*Thermoanaerobium lactoethylicum* (99%)
47-07-P	26	32	16	20	0	0	20	32	*Thermoanaerobacterium islandicum* (98%)
52-07-P	24	30	17	20	0	0	22	29	*Thermoanaerobacterium species* (98%)
9-07-X	3	6	6	10	7	6	15	19	*Clostridium hveragerdense* strain AK14 (93%)
10-07-X	10	7	12	12	11	15	24	34	Bad sequence
35-07-X	2	4	9	13	14	16	26	32	*Thermoanaerobacterium saccharolyticum* (99%)
44-07-X	21	26	14	15	0	0	18	22	*Thermoanaerobacterium islandicum* (99%)
65-07-P	1	44	8	17	13	1	24	29	Bad sequence

Unfortunately several of the potentially good ethanol producing enrichments gave bad sequences in 16S rRNA analysis. However, successful sequences showed that most of the bacteria producing large amounts of ethanol from glucose were closely related to *Caloramator* and *Clostridium* and on xylose, species within the genera of *Thermoanaerobacter*, *Thermoanaerobacterium* and *Clostridium* had the best ethanol producing capacity. Interestingly, many strains enriched on pectin and especially xylan

produced butyrate as main end product from glucose and xylose. Most of the the enrichments that produced more than 0.7 mol-H_2/mol-glucose and xylose belong to *Thermoanaerobacterium* species (Table 2 and 6).

3.3.2. End product formation from 60°C-enrichments

One enrichment culture (66-07-G) at 60°C produced more than 1.0 mol-ethanol/mol-glucose but many cultures produced between 0.70 to 0.84 mol-ethanol/mol glucose (29-07-G, 54-07-P, 64-07-P, 66-07-P, 67-07-P, 24-07-X, 34-07-X,64-07-X; Tables 3 and 6). All these enrichments showed close 16S rRNA similarity to bacteria within *Thermoanaerobacterium* except for 29-07-G who belongs to *Thermoanaerobacter*. Four enrichment cultures produced one mol or more than 1.0 mol-ethanol/mol xylose (29-07-G, 66-07-G, 66-07-P, 67-07-P) but four others produced slightly lower amounts (between 0.60 mol to 0.78 mol-ethanol/mol xylose) i.e. 8-06-Glu, 21-07-Xyl, 24-07-X and 34-07-X. 16S rRNA analysis of these high ethanol producing enrichment cultures indicated that most of them belong to either *Thermoanaerobacter* or *Thermoanaerobacterium* (Fig. 3; Table 6).

Table 3. The main phylogenetic and physiological data of enrichment cultures from 60°C. End product formation from 50 mM of glucose and xylose in mM concentrations are shown as well as the closest phylogenetic position of the enrichment in percentages.

Sample	Ethanol (mM)		Acetate (mM)		Butyrate (mM)		H2 (mmol L-1)		Phylogeny
Substrates	Glucose	Xylose	Glucose	Xylose	Glucose	Xylose	Glucose	Xylose	
8-06-G	24	31	15	17	0	0	25	23	*Thermoanaerobacterium saccharolyticum* (97,6%)
29-07-G	35	52	3	3	0	0	10	3	*Thermoanaerobacter* species (98-99%)
63-07-G	13	11	9	10	0	0	1	9	*Thermoanaerobacter* species (97 – 99%)
64-07-G	24	31	3	4	0	0	17	12	Not analysed
66-07-G	52	65	18	17	0	0	14	18	*Thermoanaerobacterium* species (98 – 99%)
21-07-Xo	31	30	18	16	0	0	34	33	*Thermoanaerobacterium saccharolyticum* (99%)
54-07-Xo	1	1	1	1	0	0	0	0	*Thermoanaerobacterium* species (98 – 99%)
21-07-Cpa	19	11	13	45	0	0	10	2	*Caloramator viterbiensis* (97%)
29-07-Cpa	12	12	38	20	0	1	1	4	*Thermoanaerobacter wiegelii* (100%)
63-07-Cpa	16	4	17	12	0	0	2	0	*Clostridium thermocellum* (92%)
21-07-Cpo	19	11	13	45	0	0	10	2	*Clostridium inferii* (95%)
29-07-Cpo	12	11	39	20	0	2	1	4	Not analysed
54-07-P	39	25	21	14	0	0	16	25	*Thermoanaerobacterium* species (98 – 99%)
64-07-P	36	27	16	13	0	0	16	13	*Thermoanaerobacterium islandicum* (95%)
66-07-P	41	49	24	20	0	0	28	26	*Thermoanaerobacterium* species (98-99%)
67-07-P	42	50	18	5	0	0	27	6	*Thermoanaerobacterium* species (95-99%)
24-07-X	39	39	15	14	0	0	24	18	*Thermoanaerobacterium* species (97 – 99%)
27-07-X	27	24	17	10	0	0	23	12	*Thermobrachium celere* (95%)
34-07-X	41	33	19	16	14	16	27	19	*Thermoanaerobacterium* species (98 – 99%)
64-07-X	37	47	31	24	0	1	15	16	*Thermoanaerobacterium islandicum* (99%)

Interestingly, enrichment culture 34-07-X produced large amounts of butyrate from both glucose and xylose as well as ethanol and acetate. Other enrichments at 60°C produced only negligible amounts of butyrate. Enrichment 21-07-Xo was the best hydrogen producer with 0.88 and 0.86 moles of hydrogen from glucose and xylose, respectively.

3.3.3. End product formation from 70 and 75°C-enrichments

Only two enrichments from 70°C (both belong to *Thermoanaerobacter*) produced high amounts of ethanol from the two mono-sugars tested; 33-07-Xo and 20-07-X (Tables 4-6).

Table 4. The main phylogenetic and physiological data of enrichment cultures from 70°C. End product formation from 50 mM of glucose and xylose in mM concentrations are shown as well as the closest phylogenetic position of the enrichment in percentages.

Sample	Ethanol (mM)		Acetate (mM)		H2 (mmol L-1)		Phylogeny
Substrates	Glucose	Xylose	Glucose	Xylose	Glucose	Xylose	
2H-06-G	16	24	2	20	11	20	*Thermoanaerobacter inferii* strain AK14 (98%)
20-07-G	2	1	14	15	23	18	*Thermoanaerobacter* species (98%)
33-07-Xo	50	43	3	4	4	7	*Thermoanaerobacter* species (98 - 99%)
55-07-Xo	8	8	7	7	9	12	*Thermoanaerobacter* species (98 - 99%)
8-07-Cpa	14	1	18	11	31	3	*Caldicellulosiruptor* species (97-99%)[1]
8-07-Cpo	2	1	16	12	10	2	*Caldicellulosiruptor kronotskiensis* (96%)
9-07-CPo	12	11	39	20	32	13	Not analysed
20-07- Cpo	13	17	7	10	6	6	*Thermoanaerobacter* species (97-98%)
4-07-X	2	3	13	13	21	19	*Caldicellulosiruptor acetigenus* clone 2 (100%)
20-07-X	39	37	2	6	9	15	*Thermoanaerobacter* species (99%)

The carbon flow is clearly directed more towards the production of acetate and hydrogen at both 70 and 75°C. At 75°C, less than 10 mM of ethanol were produced from glucose and xylose.

Table 5. The main phylogenetic and physiological data of enrichment cultures from 75°C. End product formation from 50 mM of glucose and xylose in mM concentrations are shown as well as the closest phylogenetic position of the enrichment in percentages.

Sample	Ethanol (mM)		Acetate (mM)		H2 (mmol L-1)		Phylogeny
Substrates	Glucose	Xylose	Glucose	Xylose	Glucose	Xylose	
14-07-G	1	1	2	12	0	11	Not analysed
32-07-G	1	1	1	1	0	0	Not analysed
39-06-G	1	1	12	12	20	11	*Thermoanaerobacter keratinophilus* (97%)
14-07-Xo	5	9	11	14	16	17	*Thermoanaerobacter* species (98 - 99%)
14-07-P	3	4	16	15	27	31	Not analysed
14-07-X	2	3	15	21	21	25	*Thermoanaerobacter* species (98 - 99%)

Table 6 summarizes the best ethanol and hydrogen producing bacteria with the focus upon ethanol and hydrogen production yield. The maximum yield of ethanol and hydrogen from glucose are two and four moles, respectively. The maximum yield of ethanol from xylose is 1.67 (Nielsen, Mathrani, & Ahring, 1993)

Table 6. The ethanol and hydrogen yield from selected enrichment cultures given in mol-ethanol/mol glucose and xylose and mol-H_2/mol glucose and xylose.

Temperature (°C)	Enrichment	Ethanol yield mol EtOH-mol Glucose	Ethanol yield mol EtOH-mol Xylose	Ethanol/Acetate ratio	Hydrogen yield mol H2-mol Glucose	Hydrogen yield mol H2-mol Xylose
50	2-07-G	0,42	0,64	1,0	0,78	0,71
50	15-06-G	1,32	0,62	4,1	0,57	0,52
50	44-07-G	0,56	1,02	1,7	0,55	0,59
50	44-07-Xo	0,70	0,78	1,7	0,62	0,75
50	47-07-Xo	0,50	0,32	1,6	0,57	0,47
50	65-07-Xo	0,14	1,14	0,9	0,52	0,36
50	25-07-CPa	1,34	0,34	4,5	0,44	0,39
50	35-07-Cpa	0,94	0,12	2,8	0,49	0,21
50	2-07-Cpo	0,80	0,62	2,7	0,21	0,31
50	44-07-P	0,50	0,42	1,2	0,81	0,59
50	47-07-P	0,52	0,64	1,6	0,52	0,83
50	52-07-P	0,48	0,60	1,4	0,57	0,75
50	10-07-X	0,20	0,14	0,8	0,62	0,88
50	35-07-X	0,04	0,08	0,2	0,68	0,83
50	65-07-P	0,02	0,88	0,1	0,62	0,75
60	29-07-G	0,70	1,04	11,7	0,26	0,08
60	66-07-G	1,04	1,30	2,9	0,36	0,49
60	21-07-Xo	0,62	0,60	1,7	0,88	0,86
60	54-07-P	0,78	0,50	1,9	0,42	0,65
60	64-07-P	0,72	0,54	2,2	0,42	0,34
60	66-07-P	0,82	0,98	1,7	0,73	0,67
60	67-07-P	0,84	1,00	2,3	0,71	0,16
60	24-07-X	0,78	0,78	2,6	0,62	0,47
60	34-07-X	0,82	0,66	2,1	0,71	0,49
60	64-07-X	0,74	0,94	1,2	0,39	0,42
70	33-07-Xo	1,00	0,86	16,7	0,11	0,18
70	8-07-Cpa	0,28	0,02	0,8	0,81	0,08
70	9-07-CPo	0,24	0,22	0,3	0,83	0,34
70	20-07-X	0,78	0,74	19,5	0,23	0,39
75	14-07-P	0,06	0,08	0,2	0,71	0,81
75	14-07-X	0,04	0,06	0,1	0,55	0,65

4. Discussion

In the past three decades the use of thermophilic bacteria for the production of hydrogen and ethanol has increased substantially for many reasons. The increased "food versus fuel" debate has though been an important factor. Most of the bioethanol produced today is produced from easily degradable biomass feedstock, i.e. corn in US and sugarcane in Brazil and S-America (American Coalition for Ethanol, 2009). There is an increased pressure to utilize complex lignocellulosic biomass to a greater extent for bioethanol and biohydrogen production (Koskinen, et al., 2008a). Lignocellulosic material consists of cellulose, hemicelluloses and lignin. For a complete utilization of the hemicelluloses fraction of lignocellulosic material it is necessary to use microbes that can degrade all the various monosugars that are released after pretreatment and hydrolysis (Sommer, Georgieva, & Ahring, 2004). Thus, an increased effort has been done to genetically engineer the yeast that are responsible for more than 98% of the ethanol produced from starch and sugar based livestock today (Dien, Cotta, & Jeffries,

2003). Instead of using yeast for fermentation of complex sugars derived from complex biomass it has been speculated that thermophilic bacteria might be the better choice (Dien, Cotta, & Jeffries, 2003) (Wiegel, 1980). The main reason for this is because thermophilic bacteria can degrade a broad variety of pentoses, hexoses and disaccharides. The main drawback of using thermophilic bacteria is the ethanol yield as compared to yeast fermentation. Generally, the yield of ethanol is much lower and in the range of 0.8 to 1.3 moles of ethanol for C_5 and C_6 sugars (Lacis & Lawford, 1988). This is caused by the production of other end products in the fermentation e.g. acetate, butyrate and lactate. Additionally, thermophilic bacteria have lower tolerance for ethanol as compared to yeast (Wiegel, 1980). Many thermophilic bacteria are known to produce ethanol and hydrogen from carbohydrates, especially within the genera of *Clostridium*, *Thermoanaerobacter*, *Thermoanaerobacterium*, *Caloramator*, *Caldicellulosiruptor* and *Thermotoga* (Rainey, Tanner, & Wiegel, 2006) (Cook, et al., 1996) (Kublanov, et al., 2007) (Seyfried, et al., 2002) (van Niel, et al., 2002). However, most studies on these bacteria have focused upon their phylogenetic status, not on their ethanol or hydrogen production capacity. Additionally, many studies have been directed towards their thermostable enzymes that could be used in the biotechnology industry (Collins, Gerday, & Feller, 2004). Here, a large survey of bioprospecting both ethanol and hydrogen producing bacteria from Icelandic geothermal areas varying in temperatures is presented.

In present study 60 enrichment samples were analysed for partial 16S rRNA analysis from various hot springs in Iceland. Forty nine samples revealed good sequences and the data was used for phylogenetic comparison. More than two thirds of the samples revealed bacteria that belong to the genera of *Thermoanaerobacter* and *Thermoanaerobacterium*. Clearly, the temperature is a major factor concerning the isolation of species within these two genera. All of the 19 *Thermoanaerobacterium* species were isolated from hot springs at 50 – 60°C but the majority of the *Thermoanaerobacter* species were isolated at 70 – 75°C (9 out of 12 strains).

The genera *Thermoanaerobacter* and *Thermoanaerobacterium* belong to the family Thermoanaerobiaceae and fall into the clusters V, VI and VII in phylogenetic interrelationships of *Clostridium* species (Collins, et al., 1994). The taxonomy of this group was revised by Lee and coworkers (Lee, et al., 1993). Members of *Thermoanaerobacter* and *Thermoanaerobacterium* have been isolated from unique areas e.g. deep surface oil wells (Cayol, et al., 1995), geothermal water outlets (Cook, et

al., 1996), hot springs (Wiegel & Ljungdahl, 1981), leachate of waste pile from a canning factory (Cann, et al., 2001), and sediment of hydrothermal vents (Slobodkin, et al., 1999). The difference between *Thermoanaerobacter* and *Thermoanaerobacterium* has not been very clear. Both genera have members that stain Gram positive and Gram negative (Lee, et al., 1993) (Kim, et al., 2001). Spore formation is not an effective taxonomic characteristic and the variation in this characteristic between the two genera supports this further. Previously the ability of reduce thiosulfate to sulfide (*Thermoanerobacter*) or to elemental sulfur (*Thermoanaerobacterium*) was used to distinguish between the two genuses. Cann and co-workers however proposed an emendation of the genus *Thermoanaerobacterium* since *Thermoanaerobacterium zeae* could not reduce thiosulfate at all (Cann, et al., 2001). Members of both genera are strict anaerobes. The products from glucose fermentation yields ethanol, acetate and CO_2 as well as lower yields of hydrogen, formic acid and lactate. Recently, there have been modification of the genera. *Thermoanaerobacterium fijiensis, T. zeae* and *T. polysaccharolyticum* have for example been proposed to form a new genus, *Caldanaerobius* (Lee, et al., 2008). Today, the genus *Thermoanaerobacterium* consists of 8 species and *Thermoanaerobacter* of 23 species.

The *Caloramator* genus consists of seven thermophilic bacteria; *C. fervidus* (Patel, et al., 1987), *C. coolhaasii* isolated from thermophilic methanogenic granular sludge (Plugge, Zoetendal, & Stams, 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. australicus*, isolated from the Great Artesian Basin, Australia (Ogg & Patel, 2009) and *C. uzoniensis* (Kevbrin, et al., 2002). 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). None of the species within the genus has been described as particulary powerful ethanol or hydrogen producer. *Caloramator viterbensis* has the interesting property of producing 1,3-propanediol from glycerol (Seyfried, et al., 2002). Three of the lower temperature enrichments were phylogenetically closest to *Caloramator viterbensis*.

Eleven of the samples from 50 to 60°C samples were analyzed to be bacteria that belong to three genera, namely *Clostridium*, *Caloramator* and *Paenibacillus*. At higher temperatures no enrichments were observed that belong to these genera. The *Clostridium* species isolated were most closely related to *Clostridium hveragerdense and C. thermocellum. Clostridium hveragerdense* was indeed isolated from the same geothermal area (Orlygsson & Baldursson, 2007), as samples in present study. The three isolates that were closely related to *Caloramator* showed closest relationship with *C. viterbensis* and (between 96.6 to 97.2% homology) but full 16S rRNA analysis is needed to determine if the enrichments contain bacteria that are new species.

Before the use of 16S rRNA analysis for classification of prokaryotes the genus *Clostridium* was defined as bacteria that were non-sulfate reducing, spore forming and anaerobic with Gram positive cell wall. The family Clostridiaceae contains the genus *Clostridium* (sensu strict), as well as many other genera, e.g. *Acetivibrio*, *Acidaminobacter*, *Anaerobacter*, *Caloramator* and more (Rainey, Tanner, & Wiegel, 2006). The reason for these additional genera in the family Clostridiaceae is often based upon the fact that the type species of these genera are often phylogenetically related to misclassified species of the genus *Clostridium*. However, many of these genera fall outside what is considered to be the "true Clostridiaceae" family and are now regarded to belong to other families within the low G+C Gram-positive phylum. In the last ten years our view of bacterial classification has greatly changed because of the use of 16S rRNA analysis for classification of bacteria. The phylogenetic analysis of Collins and coworkers (Collins, et al., 1994) was the first time when a substantial number of *Clostridium* species were compared both within species belonging to the genus and to related taxa. The fact that the species of the genus *Clostridium* did not form a monophyletic group has been shown in many studies (Tanner, et al., 1981) (Hutson, Thompson, & Collins, 1993) (Lawson, et al., 1993) (Rainey & Stackebrandt, 1993) (Rainey, et al., 1993) (Willems, Amat-Marco, & Collins, 1996). These investigations lead to the conclusion that more than half of the species currently assigned to the genus *Clostridium* are in fact not closely related to the type species *C. butyricum* and should therefore not be included in the newly defined genus *Clostridium*. The genus *Clostridium* comprises more than 150 validly described species (see the List of Bacterial Names with Standing in Nomenclature (Euzéby, 2009)). On the basis of 16S rRNA only 73 of these species fall within the radiation of the type species of the genus *Clostridium*, *C. butyricum* and the cluster I as defined by Collins and coworkers

(Collins, et al., 1994). The remaining species fall within the various phylogenetic clusters throughout the low G+C Gram-positive phylum. Additionally, the genus *Clostridium* (sensu strict, cluster I) has members of other valid genera and in two cases the type species of validly described genus clustering within its radiation (*Anaerobacter* and *Eubacterium*).

Three samples were classified to belong to the genus *Paenibacillus*. The optimum growth rate for the majority of the members within this genus has been reported to be 28-30°C (Shida, et al., 1997) although several species have been described to grow at slightly higher temperatures (Wang, et al., 2008). Members of this genus are aerobic, or facultatively anaerobic and several species have been reported to produce ethanol from sugar fermentation (Marwoto, et al., 2004).

From samples enriched at 70°C three samples were analysed for bacteria that belong to *Caldicellulosiruptor*. Members within this genus have been reported to be cellulolytic and good hydrogen (and acetate) producers (Bredholt, et al., 1999) (van Niel, et al., 2002). Today, seven species are phylogenetically classified within this genus and are mainly of hot spring origin. *Caldicellulosiruptor kristjanssonii, C. lactoaceticus* and *C. acetigenum* have been isolated from the same geothermal area wherefrom samples in current study focuses upon, i.e. Hveragerdi in SW of Iceland (Nielsen, Mathrani, & Ahring, 1993; Mladenovska, Mathrani, & Ahring, 1995)(Bredholt, et al., 1999). Two of the strains (*C. kristjanssonii* and *C. acetigenum*) direct their end product formation from carbohydrates to acetate, carbon dioxide and hydrogen whereas lactate is the main product from *C. lactoaceticum*. Strain 4-07-X was most closely related to *C. kristjanssonii* and *C. acetigenus* (99.5 and 99.7%, respectively) and predominently acetate and hydrogen producer. The other two samples (8-07-Cpa and 8-07-Cpo) were also most closely related to these two species although sample from enrichment 8-07-Po was not good for sequencing. Both strains were mainly producing acetate but enrichment 8-07-Pa was also producing substantial amounts of ethanol from glucose (Table 3). The other four known species within *Caldicellulosiruptor* are *C. saccharolyticus*, isolated from hot spring in New Zealand (Rainey, et al., 1994), *C. owensis*, isolated from sediment (Huang, et al., 1998), *C. kronotskyensis* and *C. hydrothermalis*, both isolated from thermal springs in Kamchatka (Miroshnichenko, et al., 2008). *C. saccharolyticus* has been extensively studied with respect to hydrogen production (van Niel, et al., 2002) (van Niel, Claassen, & Stams, 2003) but *C.*

owenensis produces a mixture of acetate, lactate, ethanol, carbon dioxide and hydrogen from carbohydrates (Huang, et al., 1998). Lactate was not analysed in present study.
Ethanol and hydrogen production from complex lignocellulosic material and mono- and disaccharides by thermoanerobes has been addressed lately. The reason for this is mostly because of the vast biomass available in the lignocellulosic material on Earth today as well as the fact that both economic and environmental issues have been debated of 1st -generation ethanol production from starch and sugar based biomass (Bullock, 2002; Balat, Balat, & Öz, 2008). Many of the strains isolated in present investigation produced considerable amounts of ethanol from glucose and xylose. Most of the "high ethanol" producing species belong the genera of *Thermoanaerobacterium* and grow at temperatures between 50 to 60°C. Additionally, several of the enrichment cultures that belong to *Clostridium, Caloramator* and *Paenibacillus* produced up to 1 mol of ethanol from one mole of glucose. The three *Caldicellulosiruptor* species produced mostly acetate and hydrogen, a well known property within members of this genus (Nielsen, Mathrani, & Ahring, 1993; Mladenovska, Mathrani, & Ahring, 1995)(Bredholt, et al.,1999).

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6. Manuscript II

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

Hydrogen and ethanol production from sugars and complex biomass by two *Thermoanaerobacterium* species isolated from Icelandic hot spring

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Abstract

Microbial fermentations are potential producers of sustainable energy carriers. In this study, ethanol production was studied by thermophilic bacterium, AK_{66}, isolated from geothermal spring in Iceland. The strain is closely related to *Thermoanaerobacterium aciditolerans* (99.1%) based on the 16S rRNA gene sequence analysis. Extensive physiological studies were done on strain AK_{66} concerning T_{opt}, pH_{opt}, substrate spectrum and yields of ethanol per mol-glucose degraded. The strain has a temperature and pH optimum at 60°C and 5.0, respectively. In batch cultivation, strain AK_{66} produced ethanol from glucose and xylose fermentation of up to 1.3 mol-EtOH/mol (65% of the theoretical maximum) glucose and 1.25 mol-EtOH/mol-xylose (75%), respectively. The strain could grow in the presence of 3.2% (vol/vol) of ethanol but not at 6.4%. During exponential growth on glucose the growth rate was 1.91 h and ethanol was produced at the rate of 2.5 mM h^{-1}. The strain was highly saccharolytic and apart from glucose and xylose it grew on arabinose, fructose, galactose, mannose, lactose, sucrose, pectin and xylan. Analyzed end products from all substrates were ethanol, acetate, hydrogen and carbon dioxide. The influence of different initial glucose concentrations on end product formation indicated a substrate inhibition at glucose loading at 50 mM. Growth of strain AK_{66} was also investigated on hydrolysates (0.75% w/v) made from cellulose (Whatman paper), newspaper, hemp leaves and fibers (*Cannabis sativa*), barley straw (*Hordeum vulgare*) and grass (*Phleum pratense*). The biomass was pretreated with heat only (121°C, 30 min) and with heat and acid (H_2SO_4, 0.75%) and base (NaOH, 0.75%). After pretreatment enzymes (CelluclastR and novozymes 188) were added prior to fermentation. End product formation was analyzed from the fermentation and compared to another well known ethanol producer, strain AK_{17}. Strain AK_{66} produced most ethanol (20 to 24 mM) from cellulose but acid and base pretreatment did not enhance ethanol yields. On other substrates, lower ethanol yield were observed (lowest on barley straw; 4 mM, highest on hemp stem (12 mM) but were increased significally by pretreatments. Yields of ethanol by strain AK_{17} were considerably higher, ranging from 5 mM on hemp leaves to 50 mM on pure cellulose. Acidic pretreatment enhanced yields mostly on hemp leaves (5 mM to 18 mM) and base pretreatment on grass (20 mM to 28 mM).

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

1. Introduction

With the inevitable depletion of the world's oil supplies, there has been an increasing worldwide interest in alternative sources of fuels (Kerr, 1998; Zaldivar, et al., 2001; John, 2004; Schubert, 2006, Sánchez & Cardona, 2008). Apart from the depletion, concerns are also on the environmental issuses concernig the use of fossil fuels (Sánchez & Cardona, 2008). Bioethanol, produced from biomass, has gained increased interest in recent years and more than 51.000 million liters are produced annually worldwide (Renewable Fuel Association, 2009). Many countries are implementing programs for the addition of ethanol to gasoline, for example in Brazil 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). First generation ethanol production in Brazil, India and South Africa is mainly based on sugar cane but in USA from corn (Zaldivar *et al.*, 2001).

Ethanol can be produced microbiologically through fermentation from various starch- and sugar-based materials (Hawkes *et al.*, 2002) but has been strongly debated the last few years since this biomass is competing with the food and feed applications. Fermentation from lignocellulosic biomass (e.g. wood, straw and grasses) is therefore an interesting alternative for the production of second generation bioethanol (Balat, *et al.*, 2008). Thermophiles have many advantages compared to mesophilic microorganisms in ethanol production concerning fast growth rates and their ability to degrade a broad variety of substrates, as well as less number of end products (Schönheit & Schafer, 1995; Sommer *et al.*, 2004; van Groenestijn *et al.*, 2002).

Hot springs are a potential source for hydrogen and ethanol producing microorganisms (Koskinen, et al., 2008). 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 in terms of temperature and pH were investigated. Kinetic parameters from glucose degradation were identified. The results obtained were compared to another strain already isolated in our group which has showed ethanol yield that is among the highest reported for thermophiles (Örlygsson & Baldursson, 2007).

2. Materials and methods

Media. The medium (per liter) consisted of: NaH_2PO_4 0.58 g, Na_2HPO_4 1.36 g, NH_4Cl 0.3 g, NaCl 0.3 g, $CaCl_2$ 0.11 g, $MgCl_2$ x $6H_2O$ 0.1 g, yeast extract 2.0 g, resazurin 1 mg, trace element solution 1 ml, vitamin solution 1 ml and $NaHCO_3$ 0.8 g. 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 otherwise 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_{66} was isolated, was 62°C (measured at sampling site) and pH 7.4 (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 7.0.

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 (and good hydrogen production), 5 mL of the enrichment culture was transferred into 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. *Escherichia coli* (AE000406) 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 4.0 to 9.0 with increments of 0.5 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 5.0. 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 and ethanol tolerance was tested on strain AK_{66}. Initial glucose concentration were 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_{66} to utilize different substrates was tested using BM medium supplemented with various filter sterilized substrates (20 mM or 3 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, VFA 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.

Pretreatment of biomass and hydrolysate preparation. Hydrolysates (HL) were made from different biomass: Whatman filter paper (cellulose), hemp leafs (*Cannabis Sativa L.*) (hemp L) and hemp stem fibers (hemp SF), (newspaper with ink (NPi), barley straw (BS) (*Hordeum vulgare*) and grass (*Phleum pratense*). The Whatman paper consists of 99% cellulose and was used as control. The hemp was collected fresh after harvesting. The hemp, the grass and the barley straw were dried overnight at 50°C and cut down 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 was 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 bottles and pretreated by 0.75% H_2SO_4 and 0.75% NaOH (control was without chemical pretreatment) before autoclaving (121°C) for 30 minutes . 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 they were 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 the hydrolysates were done in 7 mL BM medium in 120 mL serum bottles. The medium was supplemented with 3 ml of each hydrolysate (total liquid volume 10 mL), i.e. 7.5 g L^{-1}, but control samples contained no hydrolysate. The concentration of salts, vitamins and trace elements were kept the same as in the BM medium.

Analytical methods. Hydrogen, ethanol and volatile fatty acids were analyzed by gas chromatograph as previously described (Örlygsson & Baldursson, 2007). Glucose was analyzed by slightly changing the method from Laurentin and Edwards (2003). Liquid sample (400 yL) was mixed with 2 mL of anthrone solution (0.2% anthrone in 72% sulfphuric acid). The sample was boiled for 11 minutes and then cooled down on ice. Optical density was then measured at 640 nm.

3. Results

Isolation of bacterial strain and phenotypic characteristics. After three series of enrichment on glucose the culture liquid was diluted (tenfold dilution). End point dilution series were repeated three times before obtaining pure culture. Pure cultures were analysed for partial 16S rRNA analysis and later for full 16S rRNA analysis. Full 16S rRNA analysis showed that strain AK_{66} is a member of the genus *Thermoanaerobacterium* (Figure 1). The closest relative is *T. aciditolerans* (DSM 16487) with 99.1% homology of 1458 bp as well as 99.0% homology to several other *Thermoanaerobacterium* species e.g. *T. islandicum* (= AK_{17}), also investigated in this study (see later).

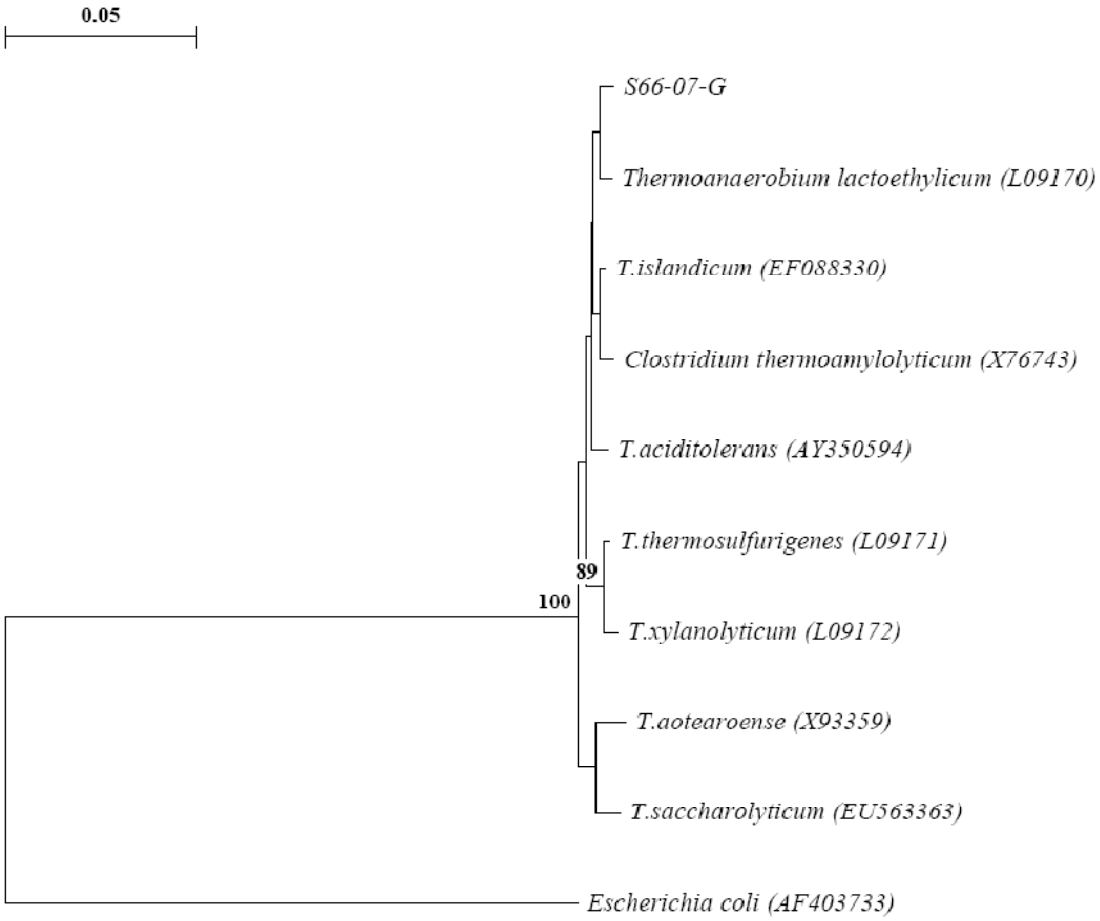

Figure 1. Phylogeny of the strains based on the 16S rRNA gene partial sequences (≈600 bp). The phylogenetic tree was generated using a distance matrix and neighbor joining algorithms with 500 bootstraps. Only supported bootstrap values (>80%) are shown. Escherichia coli (AF403733) was selected as an out-group. The scale bar indicates 0.05 substitutions per nucleotide position.

Temperature and pH ranges. The strain had a relatively broad temperature growth range or from 40.0°C to 70.0°C with T_{opt} of 60.0°C (maximum growth rate of 0.78 h^{-1} measured). No growth was observed below 40.0°C and above 70.0°C (Fig. 2A). The pH optimum was 5.0. Below pH 4.0 and above pH 7.0 no growth was observed (Fig. 2B). The maximum growth rate (μ_{max}) at pH 5.0 was 0.48 h^{-1}.

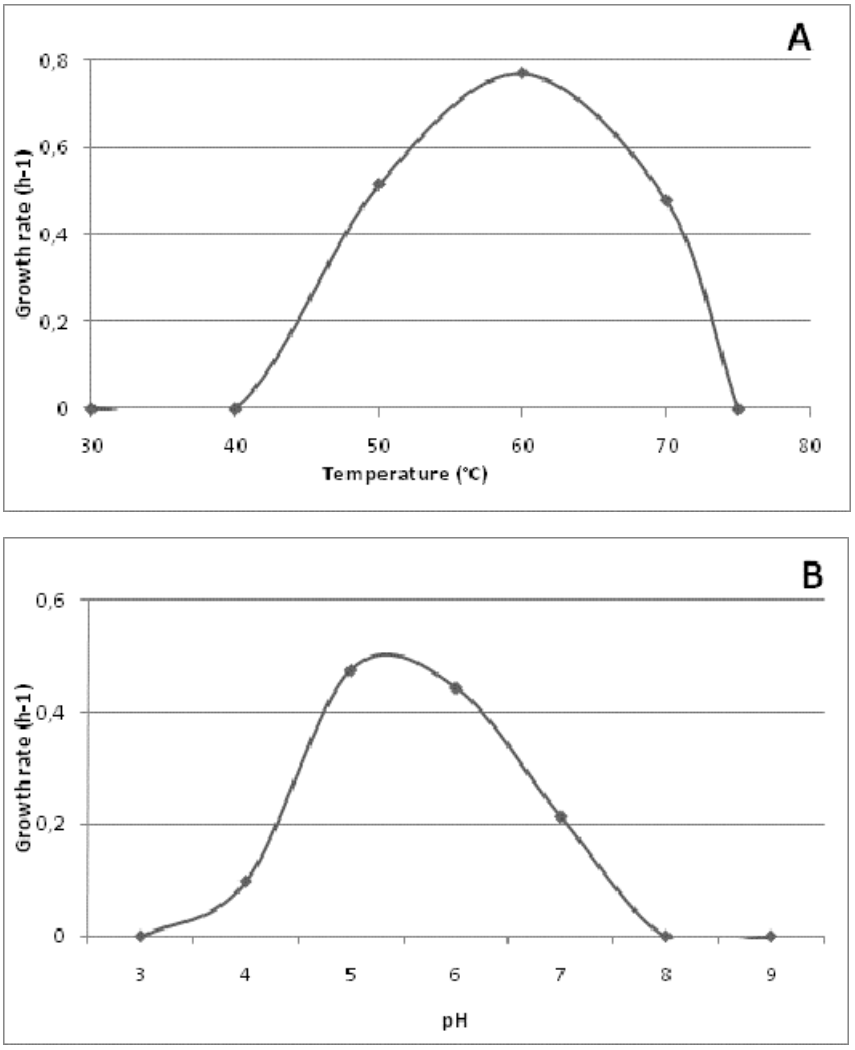

Figure 2. Optimum growth conditions for strain AK_{66}, temperature (A) and pH (B).

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

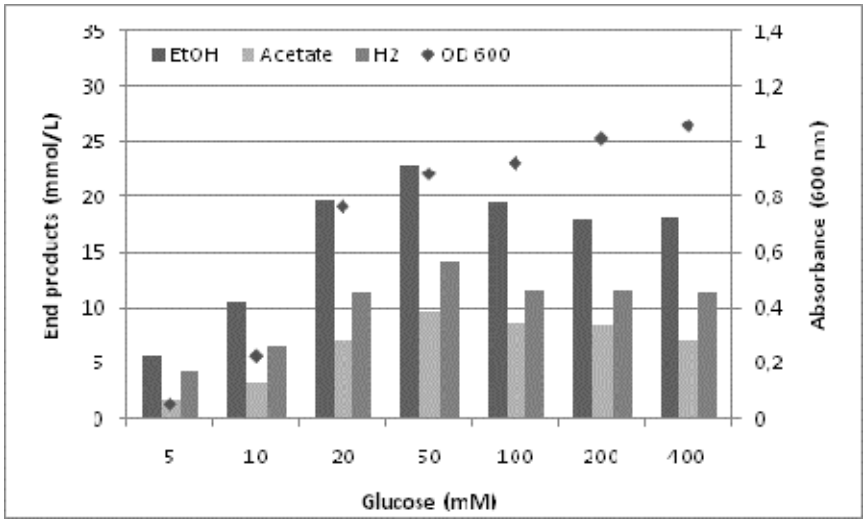

Figure 3. End product formation and growth from different substrate concentration for strain AK_{66}

At low initial glucose concentrations (5 to 20 mM) there is a relatively good correlation between the end products formed. The strain is producing approximately 1 mol-EtOH, 0.4 mol-acetate and 0.7 mol-hydrogen from one mole of glucose. However, at elevated glucose concentration, a clear inhibition of end product formation is observed. This can be seen by lower amounts of end product formation (Fig. 3) as well as insufficient glucose degradation (see later). The biomass was also measured and increased steadily from low (5 mM) substrate loadings to 20 mM (increase from 0.10 to 0.78) where after it levels off. The carbon recovery at the lower substrate concentrations (biomass excluded) ranged from 66.6% (20 mM) to 72.6% (5 mM) but were less at higher loadings. In the low (5 – 20 mM) glucose loading experiments less than 0.5 mM of glucose were left in the fermentation broth at the end of the cultivation period. At 50 mM and 100 mM, 18.5 and 78.9 mM of glucose were not degraded, respectively which explains the relatively low amounts of end products produced. Similarly, only a fraction of the glucose added at the highest loading were utilized during the fermentation time.

Substrate utilization. Of the carbon sources tested, strain AK_{66} only utilized the sugars (Fig. 4).

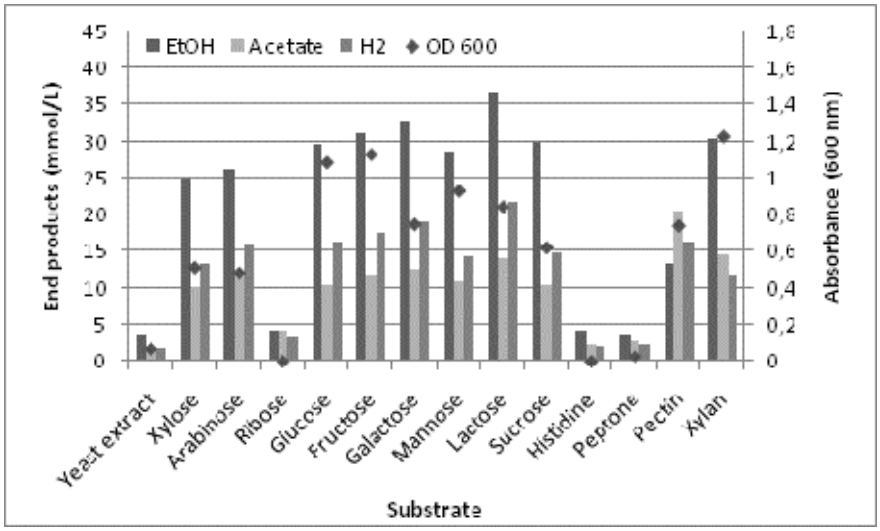

Figure 4. End product formation from different substrates for strain AK_{66}.

The strain grew well on the various pentoses, hexoses and disaccharides tested. Of the three pentoses tested, good growth was observed on arabinose and xylose but only very weak on ribose. On xylose, 21.4 mM of ethanol were produced (end products from yeast extract subtracted) which corresponds to 64% of the theoretical yield. Hexose fermentation spectrum was similar in all cases. On molar basis, between 1.24 (mannose) and 1.46 moles (galactose) of ethanol were produced from one mole of hexose degraded i.e. 62.2 to 73.3% of maximun theoretical yield. The concentration of acetate and hydrogen were approximately 30 to 50% of the ethanol concentration. Degradation of the disaccharides (sucrose and lactose) resulted in similar or slightly higher end product formation as compared to hexose degradation.

Kinetics of glucose degradation. Glucose was completely degraded in 30 hours. The doubling time was 1.91 h (μ_{max} = 0.36 h^{-1}). The main end products produced were ethanol (25.5 mM) and hydrogen (14.0 mmol L^{-1}). The ethanol production rate was 2.5 mM EtOH h^{-1} and hydrogen 2.1 mmol H_2 $L^{-1}h^{-1}$.

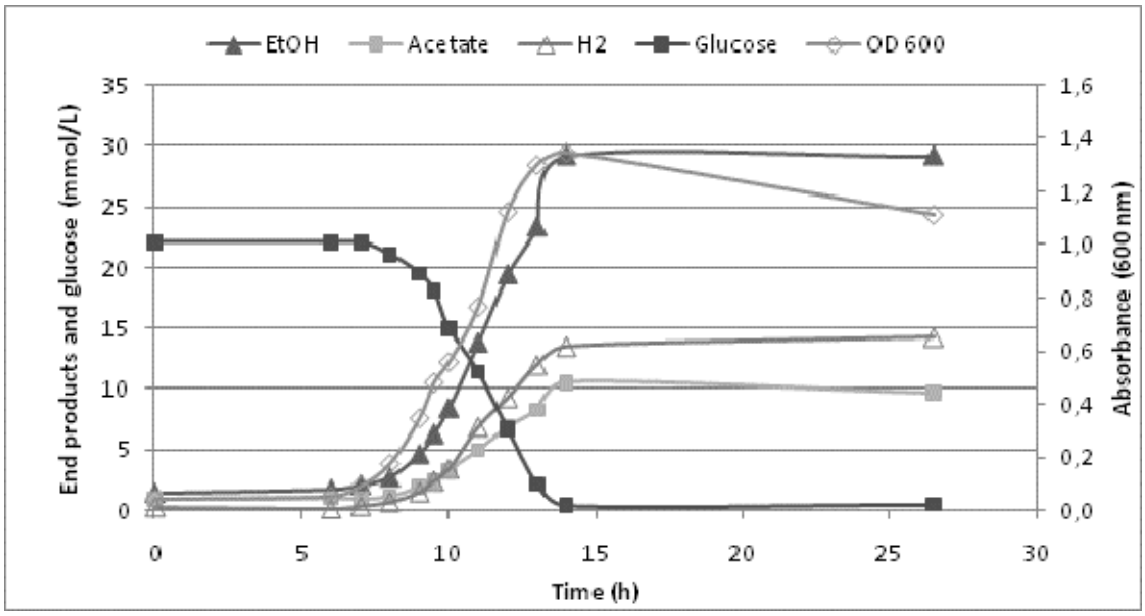

Figure 5. Growth of strain AK_{66} with glucose as carbon source;

∆= acetate, □ = ethanol, x = hydrogen, ◊ = glucose. Control sample was subtracted.

End product formation from hydrolysates

Strain AK_{66} was inoculated in BM medium containing 7.5 g L^{-1} hydrolysates from different types of complex biomass (cellulose, hemp L, hemp SF, NPi, BS and grass). Highest ethanol production was observed on cellulose (24.4 mM) but chemical pretreatment did not increase the ethanol formation from this substrate. In general the highest end product formation after cellulose was in the following order: hemp fibers > hemp leaves > grass > paper > straw. Without pretreatment the ethanol produced varied between 4.1 (straw) to 11.2 mM (hemp stem). The ethanol to acetate ratio was however much lower as compared this ratio from sugar fermentation and cellulose HL's. Additionally, the pretreatment for some of the biomass had much more effect on the ethanol yield as compared to the cellulose HL's. This is most profound in the straw HL where ethanol formation increases twofold in the acid pretreated straw and three times in the base pretreatment as compared to straw that was only heated.

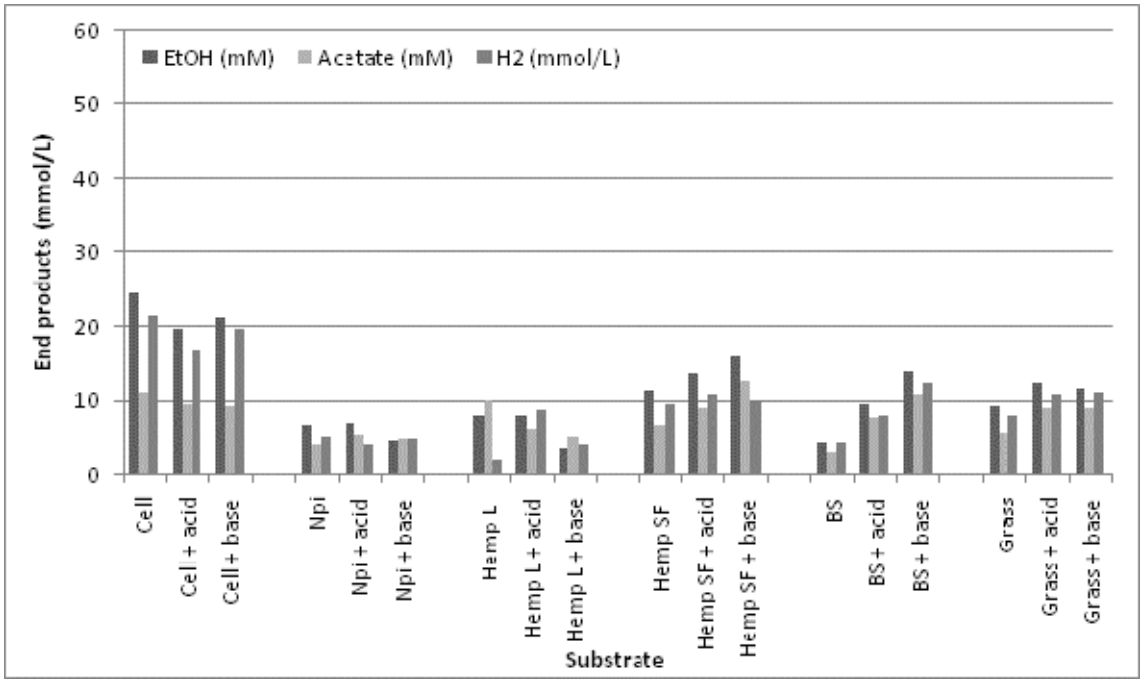

Figure 6. End product formation by strain AK_{66} from various biomass types (Cell = cellulose), Npi (non inked paper), Hemp L (hemp leaves), Hemp SF (hemp stem fiber), BS (barley straw), and Grass is Phleum pratense grass. The biomass was pretreated with acid and base.

For comparison, the HL's fermentation was also done on another strain, AK_{17}, but this bacterium has been reported to have one of the highest ethanol yields reported (Örlygsson and Baldursson, 2007). This strain produced much more ethanol compared to strain AK_{66}. On cellulose, between 40 to 50 mM of ethanol were produced, thereafter most yields were on hemp stem and grass (between 20 to 30 mM) but fermentation of untreated paper, hemp leaves and straw resulted in the production of between 5 to 15 mM of ethanol. Again, acid and base pretreatment on the various biomass usually resulted in higher ethanol yields on the complex biomass types. For example, acid pretreatment of hemp leaves gave four times higher ethanol as compared to untreated control sample and acid and base pretreatment of straw resulted in two and three times more ethanol as compared to no chemical pretreatment, respectively.

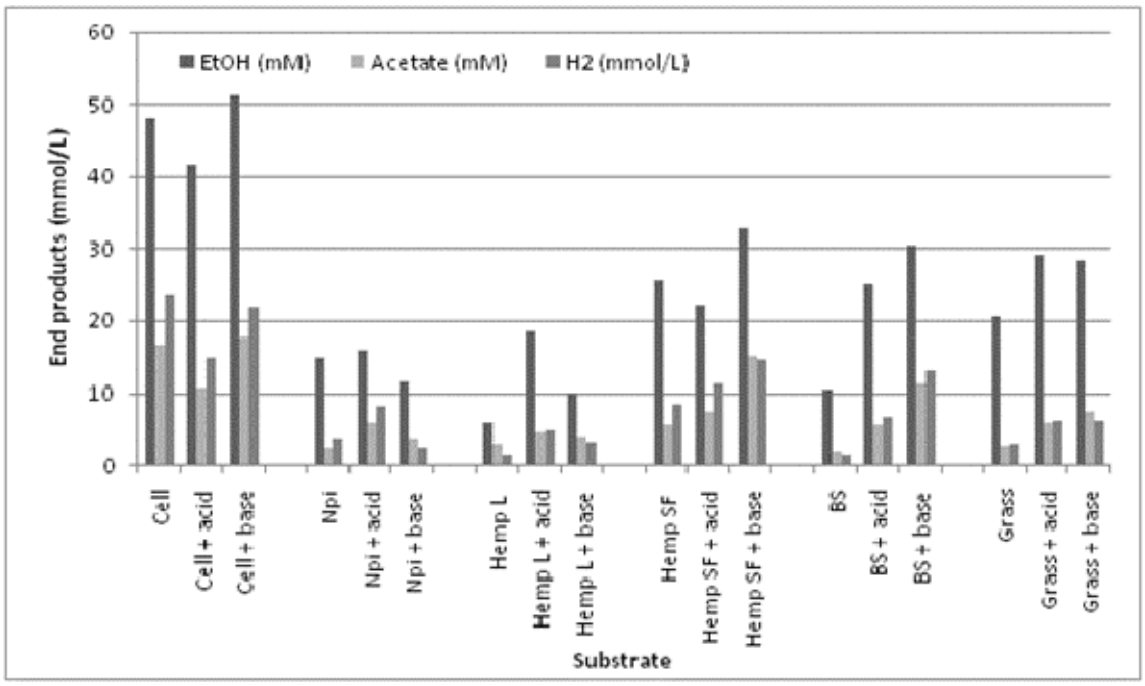

Figure 7. End product formation by strain AK_{17} from various biomass types (Cell = cellulose), Npi (non inked paper), Hemp L (hemp leaves), Hemp SF (hemp stem fiber), BS (barley straw), and Grass is Phleum pratense grass. The biomass was pretreated with acid and base.

4. Discussions

Ethanol and hydrogen producing bacteria have gained much attention the last recent years because of increased interest in renewable energy sources. High values of hydrogen produced per mol of glucose utilized have been reported from pure cultures of the hyperthermophiles *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii*; 3.3 mol-H_2 /mol-carbohydrate (van Niel *et al.*, 2002) and enrichment 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 in batch culture (Koskinen *et al.*, 2008b). Among high ethanol producing thermoanaerobe, *Thermoanaerobacter thermohydrosulfuricus* produces 1.5 mol EtOH/mol-glucose (Wiegel, Ljungdahl, & Rawson, 1979)The highest ethanol yield reported is for the *Thermoanaerobacter ethanolicus*, 1,9 mol-EtOH/mol-glucose in both batch (Wiegel and Ljungdahl, 1981) and continuous cultures (Lacis and Lawford, 1991).

Strain, AK_{66} was isolated from Icelandic geothermal area in Grensdalur, in SW-Iceland on glucose. The temperature in the hot spring from where the strain was isolated was

62.0°C and the pH 7.40. When isolated, the temperature was slightly below the natural environmental temperature, 60°C, and at pH 6.0. Thus, it was not surprising that the strain had similar optimal growth temperature as the natural environmental conditions.

Substrate concentration can play an important role in ethanol yields (van Ginkel & Logan, 2005). In present study, strain AK_{66} was cultivated on different initial concentrations of glucose varying from 5 – 400 mM. Results show that ethanol production does not increase when glucose concentration is increased (Fig. 3) from 50 mM to 100 mM. This could possibly be explained by substrate inhibition (van Ginkel & Logan, 2005) or pH inhibition. Clearly, the glucose was only partially degraded at substrate loading of 50 mM. Unfortunately, the pH was measured at the end of the incubation time.

When lignocellulosic biomass is hydrolysed the main sugars are glucose and xylose (Hamelinck *et al.*, 2005), both of which strain AK_{66} degrades. When choosing the amount of hydrolysate added to the media, 7.5 g L^{-1} was chosen. This is however slightly above the inhibition effects of substrate loading if all the glucose that is present in the Whatman paper would be released during pretreatment to glucose. 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_{66} produced 26.0, 9.5 and 12.5 mmol L^{-1} ethanol, acetate and hydrogen (control substracted from data in Fig. 5) from 20 mM glucose, respectively (Fig. 5), giving the stochiometry:

1.0 Glucose → 1.30 EtOH + 0.48 Acetate + 0.62 H_2 + 1.78 CO_2

From the cellulose HL the strain produced 19.7 to 24.4 mM ethanol, depending on the pretreatment used. Theoretically, the production could be 54.2 mM EtOH from 41.7 mM glucose. Clearly the strain does not degrade all the glucose in the hydrolysate. Unfortunately, glucose was not analysed in the culture broth in this experiment.

For comparison, strain AK_{17} was used but this strain has been reported to produce 1.5 mol-EtOH/mol-glucose (Örlygsson et al., 2007; Sveinsdóttir et al., 2009). Strain AK_{17} has been shown to have the following stochiometry for glucose fermentation:

Glucose → 1.5 EtOH + 0.5 Acetate + 0.7 H_2 + 2.0 CO_2

Thus, theoretically this strain should produce 62.5 mM of ethanol. The actual concentration found in the fermentation broth was 46.6 mM (control subtracted) or 74,6% of the theoretical yield. In an earlier experiment on various concentration of cellulose HL it has been shown that at 5 g L^{-1} the theoretical yield of ethanol was 93% but at 17.5 g L^{-1} it decreased to 22% (Sveinsdottir et al., 2009) for this strain. Thus, clearly, the substrate concentration influences strongly the efficiency of the glucose conversion to ethanol. The difference in ethanol production between strain AK_{17} and AK_{66} on various hydrolysates is surprisingly high considering the yields from glucose fermentation.

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 straw where the EtOH production increased for both strains when acid and base pretreatment was used. Strain AK_{66} produced 4.1 mM (only heat pretreatment) to 9.5 mM (acid) and 13.8 mM (base) of ethanol were used. Similar values on straw for strain AK_{17} were 10.3 (no pretreatment) to 25.0 (acid) and 30.5 mM (base). Similarly, on grass, and hemp (both leaves and stem) the general trend was a substantial increase in ethanol yields when chemical pretreatment was used. An exception of this is on hemp leaves for strain AK_{66} when base was used and on hemp stem for AK_{17} when acid was used. Pretreatment of paper however did not enhance ethanol yields to any great extend an in fact lower yields were observed in some cases which is not surprising since no hemicellulose is present. Phylogenetic studies on strain AK_{66} revealed that the bacterium belongs to the genus *Thermoanaerobacterium*. The closest phylogenetic relative was *Thermoanaerobacterium acidotolerance* (99.1% similarity), a well known ethanol and hydrogen producer (Kublanov *et al.*, 2007). Other strains closely related to strain AK_{66} are *Clostridium thermoamylolyticum*, *Thermoanaerobacterium islandicum, Thermoanaerobium lactoethylicum* (all 99.0%). All have similar phenotypic characteristics as strain AK_{66} and most of them have been reported to produce ethanol and hydrogen (U. S. Pat. 4536477; Liu *et al.*, 1996; Orlygsson & Baldursson, 2007).

5. ACKNOWLEDGEMENTS

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7. Conclusions

Thermophilic fermentative bacteria have gained increased focus recently because of their broad substrate spectrum capacity which is an important factor for utililzing lignocellulosic biomass for the production of bioethanol. In the present study 60 enrichment samples were physiologically and phylogenetically analyzed from various hot springs in Iceland. The major findings were that the majority of the samples revealed bacteria that belong to the genera of *Thermoanaerobacter* and *Thermoanaerobacterium*. Clearly, the temperature is a major factor concerning the isolation of species within these two genera. All of the 19 *Thermoanaerobacterium* species were isolated from hot springs at 50 – 60°C but the majority of the *Thermoanaerobacter* species were isolated at 70 – 75°C (9 out of 12 strains). Other bacteria at moderate temperatures showed presence of bacteria that belong to three genera, namely *Clostridium*, *Caloramator* and *Paenibacillus*. From samples enriched at 70°C three samples were analysed for bacteria that belong to *Caldicellulosiruptor*, a well know cellulolytic bacteria that are good hydrogen (and acetate) producers.

Many of the strains isolated in present investigation produced considerable amounts of ethanol from glucose and xylose. Analysis of ethanol production from all enrichments revealed that most of the "high ethanol" producing species belong the genera of *Thermanaerobacterium and Thermoanaerobacter* and grow at temperatures between 50 to 60°C. Additionally, and one of the enrichment culture that belong to *Paenibacillus* produced up to 1 mol of ethanol from one mol of glucose.

One of the most important factor concerning commercializing the use of thermophilic bacteria for ethanol production from lignocellulosic material is the substrate/sugar loadings and ethanol tolerance as well as tolerance to toxic compounds released/produced during the production of hydrolystates from lignocellulosic material. The effect of substrate loading was investigated in more detail with six strains isolated in this study and further two strains were subject to fermentation of hydrolysates from various biomass types. Clearly, the concentration of hydrolysates is a critical factor concerning the conversion of sugars to ethanol. This was indeed observed during growth of selected isolates on different concentrations of glucose. Inhibition in ethanol production was often observed at initial glucose concentrations of 50 mM or in hydrolysates between 5 to 10 g L^{-1} (dw). Thus, for scaling the process up, fed batch

cultivation or continuous culture seems to be necessary for increasing ethanol production and for complete sugar degradation. Calculations from one of the strains on hydrolysates from grass and hemp show that it is possible to produce 250 liters of ethanol from one tonn of biomass (dw).

8 Appendices