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Ethanol Production from Timothy (*Phleum pratense* L.)

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60 ECTS thesis submitted in partial fulfillment of a *Magister Scientiarum* degree in Agricultural Sciences

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Clarification of Contribution

I hereby declare that the work of this thesis, the experiments performed, and the writing is my work under the supervision and assistance of my advisors, Þóroddur Sveinsson M.Sc. and Dr. Jóhann Örlygsson. This thesis has not been submitted previously for other purposes.

Þóroddur Sveinsson harvested the timothy fields, took samples for this project, and put up the statistical analysis. Tryggvi Eiríksson at the Agricultural University of Iceland performed the hay and hydrolysate residue analysis. Dr. Jóhann Örlygsson performed measurement of ethanol and acetate concentrations of samples. Sean M. Scully performed sugar analysis with HPLC.

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Helgi Elí Hálfdánarson

Abstract

Declining petroleum stocks and the environmental effects of their usage has increased interest in alternative and renewable resources. Timothy (*Phleum pratense* L.) is an interesting option as a resource for second generation of bioethanol production. This project was set out to investigate the effect of different harvest times on ethanol yield by several ethanologens including *Clostridium thermocellum*, *Thermoanaerobacter ethanolicus*, *Thermoanaerobacter* strain J1, *Zymomonas mobilis*, *Kluyveromyces marxianus,* and *Saccharomyces cerevisiae*. The timothy samples were collected from the Möðruvellir Experimental Station in the summer 2014 at four different growth stages: vegetative stage (H1), mid-heading (H2), full-heading (H3), and post blooming (H4). The results of this research indicate that different harvest time of timothy had a statistically significant effect on ethanol yield (mM/L of timothy hydrolysate), although the differences were not significant for all harvest times or for all strains. Earlier harvest dates differed in ethanol yield more so than the latter collection periods. However, the ethanol production efficiency varied little between harvest times. The first harvest time was usually significantly different from the rest. Calculated on area bases for the best ethanol-producing strains, there was, in most cases, a significant difference in ethanol yield from timothy hydrolysates between harvest times prior to mid-heading (H1 and H2) and after full-heading (H3 and H4). *S. cerevisiae* was the best ethanol-producing strain examined in this project. *S. cerevisiae* had the highest ethanol production efficiency, 346 L/t DM from the second harvest time of timothy. The highest ethanol yield was 2,211 L/ha by *S. cerevisiae* on hydrolysate from the fourth harvest time. Given that the potential arable land for large-scale biomass production in Iceland is approximately 420 km^2, it should be possible to produce about 92.9 million L of ethanol by this method. This project revealed that the timothy solubility was from 50% to 79%, which means that the formation of hydrolysate residue is unavoidable. The possible use of the residue and the fertiliser value can only be determined with experimentation.

Keywords: Bioethanol, lignocellulosic biomass, hydrolysate, thermophiles, arable land.

Útdráttur

Þverrandi birgðir jarðefnaeldsneytis og umhverfisáhrif þess hafa aukið áhuga á öðrum endurnýjanlegum orkugjöfum. Framleiðsla lífetanóls með annars stigs gerjun á vallarfoxgrasi er áhugaverður kostur. Í þessu verkefni voru skoðuð áhrif mismunandi sláttutíma á etanól uppskeru. Örverur sem notaðar voru til etanól framleiðslunnar voru: *Clostridium thermocellum*, *Thermoanaerobacter ethanolicus*, *Thermoanaerobacter* stofn J1, *Zymomonas mobilis*, *Kluyveromyces marxianus* og *Saccharomyces cerevisiae*. Vallarfoxgrasið var slegið sumarið 2014 á Möðruvöllum í Hörgárdal. Borin voru saman fjögur þroskastig vallarfoxgrassins: blaðvöxtur (H1), mið-skriðtími (H2), eftir skrið (H3) og að lokinni blómgun (H4). Niðurstöður verkefnisins benda til þess að sláttutími vallarfoxgrass hafi marktæk áhrif á etanól uppskeru (mM/L). Það var þó ekki marktækur munur fyrir alla sláttutíma né fyrir alla örverustofna sem notaðir voru. Oft var marktækur munur á etanól framleiðslu milli snemmslegna grasins og því síðarslegna. Það reyndist ekki mikill munur á skilvirkni etanól framleiðslunnar milli sláttutíma. Það var yfirleitt marktækur munur á fyrsta sláttutímanum samanborið við hina þrjá. Þegar framleiðslan var reiknuð yfir á einingu lands (L/ha), reyndist í flestum tilfellum marktækur munur á etanól uppskeru milli sláttutíma fyrir miðskriðtíma (H1 og H2) og svo eftir skrið (H3 og H4). Sveppurinn *S. cerevisiae* skilaði bestu skilvirkni etanól framleiðslunnar á öðrum sláttutíma (H2), alls 346 L/t þe. *S. cerevisiae* skilaði jafnframt mestri etanól framleiðslu umreiknað á einingu lands, eða 2.211 L/ha. Miðað við að á Íslandi sé mögulegt ræktarland til stórframleiðslu orkujurta um 420 km^2, þá ætti að vera hægt að framleiða um 92,9 milljónir lítra af etanóli með þessari aðferð. Þá kom fram í verkefninu að leysanleiki vallarfoxgrass reyndist vera á bilinu 50-79%. Það þýðir að umtalsvert hrat verður til við framleiðsluna. Mikilvægt er að setja upp rannsóknir til að kanna hver möguleg afnot hratsins gætu verið og hvert áburðargildi þess er.

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List of Abbreviations

5-HMF: 5-Hydroxymethyl furfuraldhyde

ATP: Adenosine triphosphate

BM: Basal medium

C: Carbon

CBP: Consolidated bioprocessing

CO: Carbon monoxide

CO_2: Carbon dioxide

CORINE: Coordination of Information on the Environment

DF: Degrees of freedom

DM: Dry matter

DMD: Dry matter digestibility

GDD: Growing day degrees

GHG: Greenhouse gases

GIS: Geographic Information Systems

H1: First harvest time

H2: Second harvest time

H3: Third harvest time

H4: Fourth harvest time

HLs: Hydrolysates

MASL: Meters above sea level

N: Nitrogen

N/A: Not available

NDF: Nutrient detergent fibres

NLSI: National land survey of Iceland

NO_x: Nitrogen oxides

RCG: Reed canary grass

SE: Standard error

SHF: Separate hydrolysis and fermentation

SO_x: Sulphur oxide

SSCF: Simultaneous saccharification and co-fermentation

SSF: Simultaneous saccharification and fermentation

ST: Student's t test

YE: Yeast extract

List of Definitions

Ethanol concentration: The final ethanol concentration in bottles, measured in mM/L.

Ethanol production efficiency: The production of ethanol per unit of biomass, measured in L/t DM of timothy.

Ethanol yield: The ethanol production on area basis, measured in L/ha.

Timothy yield: The dry matter yield of timothy grass on area basis, measured in t DM/ha.

1. Introduction

Fossil fuels have been the primary source of energy for transportation, both in the 20th century and so far in the 21st century. The vast majority of vehicles are powered by the combustion of gasoline, diesel, or natural gas. Fossil fuels are a finite energy resource that is rapidly becoming scarcer and more expensive to produce. Additionally, the use of fossil fuel is the main reason for increased carbon dioxide (CO_2) emissions and anthropogenic global climate change (Balat & Kirtay, 2010). Declining petroleum stocks and the negative environmental effect of their usage has increased interest in alternative and renewable resources (Gell et al., 2011). An alternative energy resource must be technically feasible, affordable, readily available, sustainable, and environmentally benign (Balat, 2008). Available energy can be converted to usable energy in many ways and there are many options know to be referred as renewable resources for example sunlight, wind, biomass, water, and geothermal energy (Schlager & Weisblatt, 2006). Also, many different types of fuels have been proposed as alternative to fossil fuels including: biodiesel, methanol, ethanol, hydrogen, and natural gas (Balat, 2008). The subject of this study is ethanol production and therefore other options are not discussed further in this thesis.

1.1 Sustainability

In 1987 the World Commission on Environment and Development (Brundtland Commission) defined sustainable development as "development, which meets the needs of current generations without compromising the ability of future generations to meet their own needs". Economic and social prosperity cannot be amended by destroying the environment (UNECE, n.d.). It is nearly impossible to organize the sustainable utilization of a resource that will not have any impact on the environment so systems should be designed to have as little negative impact on societies and the environment as possible.

Bioethanol production from biomass is regarded as promising renewable energy source. With main advantages of lower greenhouse gas (GHG) emissions and possibly reduced NO_x and sulphur oxide (SO_x) emissions (Demirbas, 2009). Biomass is a CO_2 neutral source (Balat & Kirtay, 2010), since the CO_2 formed from the combustion of biomass products would originally have been absorbed from the air (Lin & Tanaka, 2006). A simplified scheme of the process is shown in figure 1.

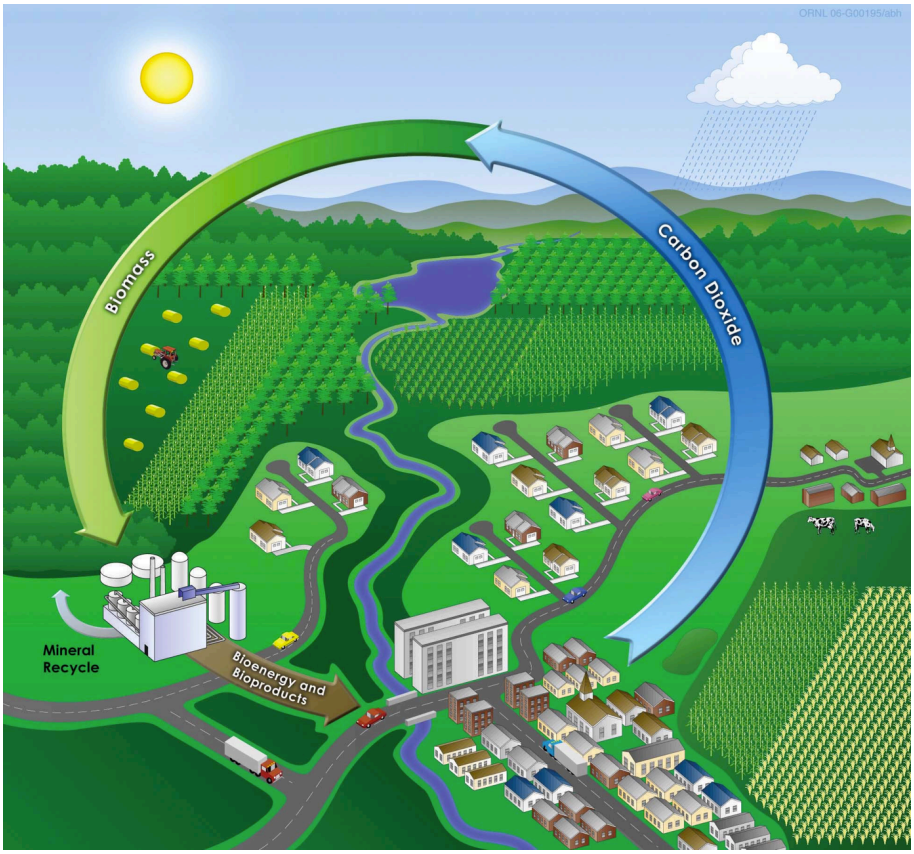

Figure 1. Reduced CO_2 emissions by ethanol from biomass (US DOE, 2007b).

1.2 Oil Production

The world's oil production has greatly increased since 1965, as shown on figure 2. The oil crises in the 1970's and early 1980s caused a reduction in oil production and oil prices increased rapidly. Gasoline appeared to be in short supply and nations began to limit public access to fuel. However, the crisis spurred a new interest in fuel economy and alternative energy resources (Schlager & Weisblatt, 2006). However, since 1981 the annual oil production has increased more than one million tonnes resulting in a decrease in price and a decreased interest in renewable alternative energy carriers.

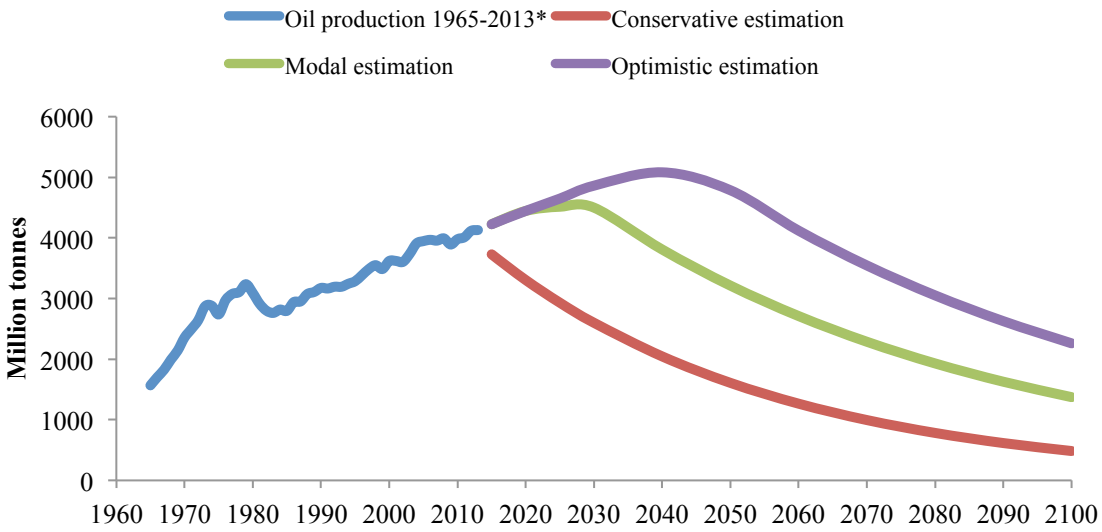

Figure 2. Annual global oil production (includes biofuels, such as ethanol, biodiesel and derivatives of coal and natural gas) *(BP, 2014), and probabilistic estimation of global oil production (the estimates performed address conventionally recoverable oil resources and reserves) until 2100 (Kontorovich, 2009).

Figure 2 shows world's total oil production from 1965 to 2013 and three different probabilistic estimates of the production until 2100. The three different estimates, conservative, modal and optimistic are based on initial recoverable oil in the year 2000 to be; 380 billion tons; 500 billion tons; and 660 billion tons, respectively. This estimation suggests that maximum oil production will be achieved in 2020-2030 (Kontorovich, 2009).

1.3 Oil Consumption in Iceland

Total oil use in Iceland has increased since 1983 (figure 3). The oil use was quite steady the first decade of this century, when oil usage decreased in the fishing industry but increased in transportation. That is mostly because of less oil usage by the fishing industry as a result of less catch during that period. Also, oil usage in some industries has been increasingly replaced by electricity usage. On the other hand, oil usage for transportation increased rapidly during that period (Orkuspárnefnd, 2008). The economic crisis in 2008 caused a rapid decline in oil usage, mostly because of less international transport. International transport started to increase again in 2011 (Orkuspárnefnd, 2012). Some decline in usage could be explained by increasing popularity of fuel-efficient cars (Eyvindsson, 2011, February 10th.).

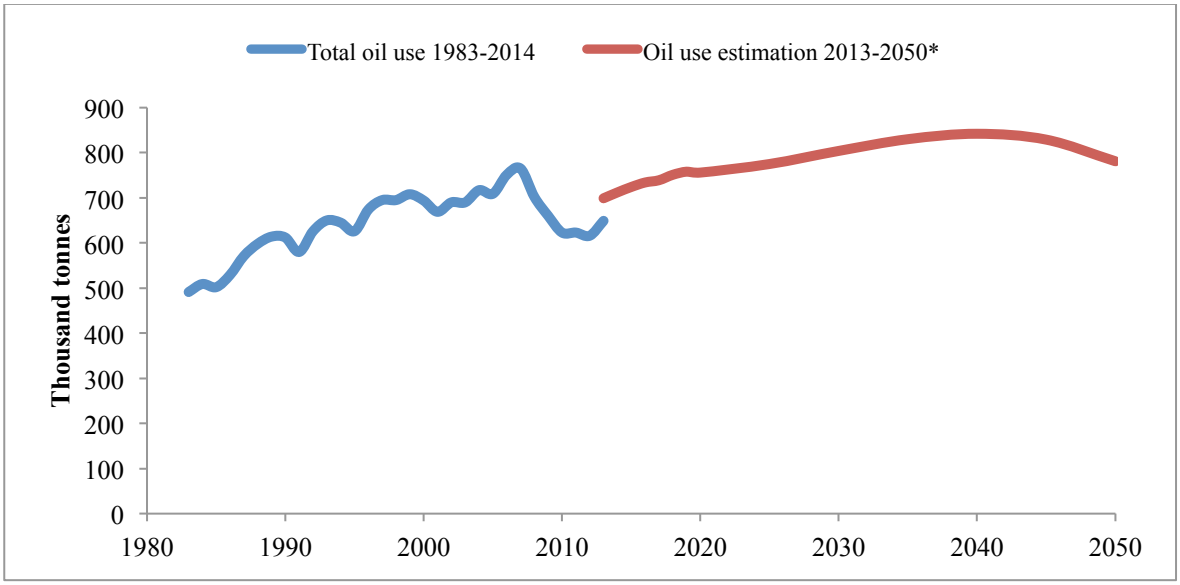

Figure 3. Total annual oil use in Iceland during the period 1983 - 2014 (Statistics Iceland, n.d.) and probabilistic estimation of total oil use 2013 - 2050 *(Orkuspárnefnd, 2008).

As shown on figure 3, it is estimated that oil use in Iceland will peak during the middle of the fourth decade of this century and then start to decline, as other energy resources will become available and replace the oil as energy source (Orkuspárnefnd, 2008).

1.4 Bioethanol

There is a long history of ethanol production via brewing. Ethanol can be used for many other purposes than just as a beverage including as a fuel, a feedstock for ethylene production (a key intermediate in the petrochemical industry), and as starting material for the manufacture of various chemicals including acetic acid, acetaldehyde, and butanol (Glazer & Nikaido, 2007).

Anhydrous ethanol was used as fuel in internal combustion engines by the late nineteenth century. There were also ideas that farmers would produce their own engine fuels and the U.S. Congress removed the tax on alcohol in 1906 in attempt to assist the project (Glazer & Nikaido, 2007). There were some that believed that ethanol made from grain would be a valuable fuel, for example Henry Ford (1863-1947). However, ethanol was not used much as fuel until the oil crisis in 1970's. Given that the world was running out of oil, ideas came up to use ethanol instead of gasoline. At first it was a small movement in the United States,

mainly in the corn-growing states but then in Brazil where it became a big industry (Schlager & Weisblatt, 2006).

The Brazilian National Alcohol Program was set out in 1975, a determined effort to replace gasoline with ethanol produced from sucrose derived directly from sugarcane. The total production of ethanol per year has increased rapidly since the program started. By 1989, Brazil produced 12 billion litres of ethanol annually, enough to drive 4.2 million cars on hydrated ethanol (95% ethanol, 5% water) and 5 million on a blend of 78% gasoline and 22% ethanol. In 1996, the production went up to 13.9 billion litres of ethanol per year (Glazer & Nikaido, 2007). In 2014, the production was up to 23.4 billion litres, according to the renewable fuels association statistics (RFA, n.d.).

The United States is today, the largest alcohol producer in the world. The Energy Security Act was passed in 1980, including the Biomass Energy and Alcohol Fuels Act, to provide loans and loan guarantees to bioethanol and biomass-related energy projects. The aim of this project is to encourage the addition of 10% alcohol to gasoline, called "gasohol". Combustion of gasohol leads to lower amounts of nitrogen oxides (NO_x) and carbon monoxide (CO) when compared to combustion of regular gasoline. In 1988, the blended gasoline was accounted for about 7% of total gasoline sales. Almost all the fuel ethanol in United States is produced by fermentation from corn. More than 12.5 billion litres were produced in 2003 and with the average yield of 0.37 L of ethanol/kg of dry corn kernels (12-15% moisture) (Glazer & Nikaido, 2007). The production went up to 54.1 billion litres in 2014, according to the renewable fuels association statistics (RFA, n.d.).

The increase in global bioethanol production during the period from 1975 to 2013 is shown in figure 4. The rapid increase in the early 21st century can both relate to concerns of limited fossil fuel supply and environmental effect from the burnt fossil fuel (Schlager & Weisblatt, 2006).

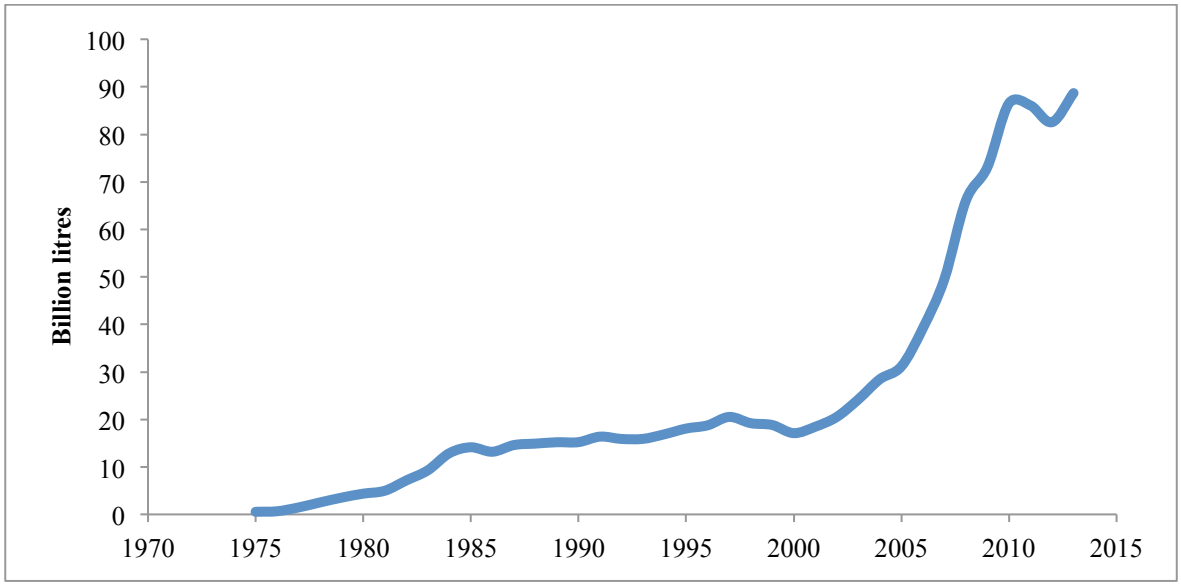

Figure 4. Global bioethanol production 1975 - 2013 (modified from: EPI, 2012; Licht FO, n.d.).

1.4.1 Food vs. Fuel Debate

Currently, a vast majority of bioethanol produced is first generation bioethanol, produced from sugarcane, sugar beet, or cornstarch (Scully & Orlygsson, 2014). Despite the success in first generation feedstocks, there are increasing concerns of using food and feed crops as feedstock for energy production. Also, to use finite arable land for biofuel production and the environmental impact of farming energy crops (Rathmann et al., 2010). These concerns are better known as the food versus fuel debate. In response to the food versus fuel debate, there is now increased focus on second generation biofuels that do not compete with food supply directly, production from non-food biomass e.g. wastes and by-products from other agricultural production (Sims et al., 2010).

1.5 Biomass

Biomass is defined as "all organic matter that grows by the photosynthetic conversion of solar energy" (Glazer & Nikaido, 2007). Therefore, it is organic material that stores sunlight as chemical energy. Biomass consists of products, by-products, residues, and waste from agriculture, forestry, and related industries; biomass is typically composed of cellulose, hemicelluloses, lignin, lipids, proteins, simple sugars, starch, water, hydrocarbons, ash, and other compounds (Demirbas, 2009). There are many types of biomass that can be used as material for ethanol fermentation. First generation bioethanol

is generated from homogenous substrates, simple sugars like sucrose from sugar cane and starch from corn. Second generation ethanol is produced from more complex (lignocellulosic) biomass. Lignocellulose is formed of three main components: cellulose, hemicellulose and lignin. These components are strongly bound together by noncovalent bonds and also covalent cross-links (Glazer & Nikaido, 2007; Scully & Orlygsson, 2014). Stages in the conversion of biomass to ethanol, from different biomass are shown in figure 5.

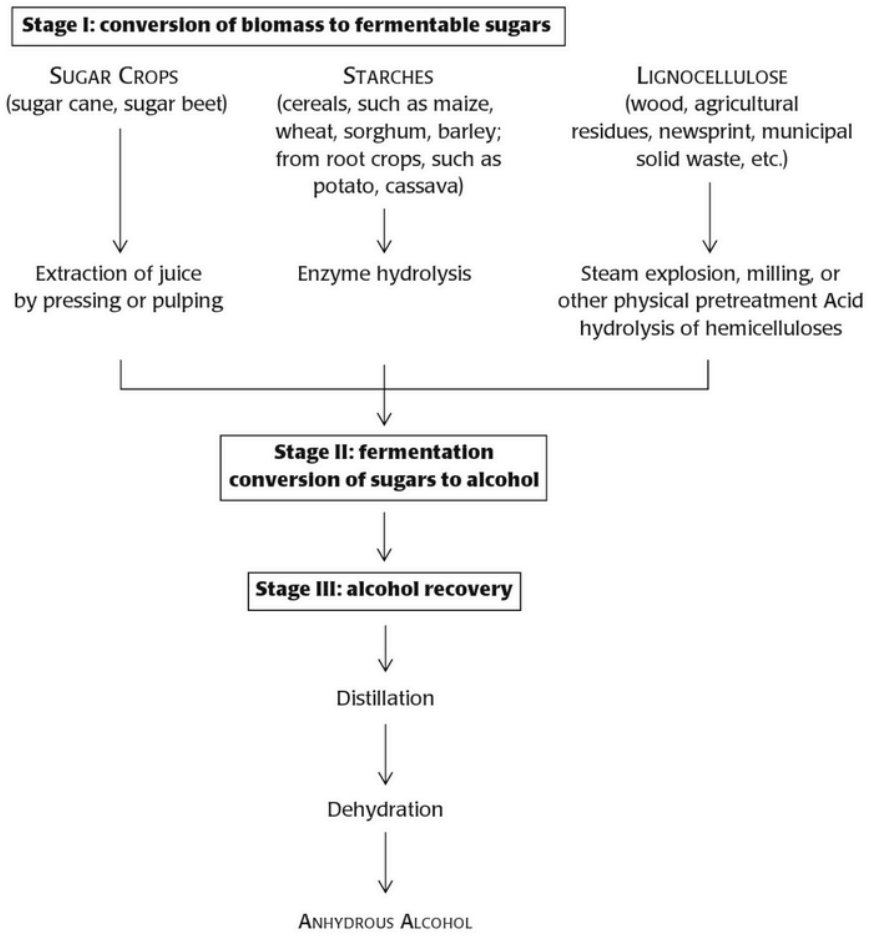

Figure 5. Stages in the conversion of biomass to ethanol (Glazer & Nikaido, 2007).

1.5.1 Sugars

Sucrose is the most common sweetener for human consumption. Both sugarcane and sugar beets contain up to 20% sucrose by weight. When sucrose is used as substrate for fermentation, the sucrose is extracted with water after the cane or beets have been mechanically crushed or stripped and pulped, respectively. Alcohol production from

sucrose found in sugar cane is especially favourable with yeast fermentation. *Saccharomyces cerevisiae* and related yeasts produces the enzyme invertase that hydrolyses the sucrose to glucose and fructose, which can then be converted to ethanol by fermentation (Glazer & Nikaido, 2007).

1.5.2 Starch

Starchy materials such as corn, potatoes, sorghum, wheat, and barley, can be used as feedstock for ethanol production. Starch is a biopolymer and defines as a homopolymer consisting of only one monomer, D-glucose. When starch is used for ethanol production, it is important to break down the chains of the carbohydrates to obtain glucose syrup. The glucose syrup can then be converted to ethanol by yeasts (Demirbas, 2009). Cornstarch is the main feedstock for alcohol production in the United States. Cornstarch contains amylose (20%) and amylopectin, which is water-soluble and water-insoluble, respectively. Heating a mixture (slurry) of milled dry corn and water produces cornstarch. The starch solubilizes when the slurry is heated, which makes the starch more acceptable for enzyme digestion. Prior to fermentation, a thermostable α-amylase is added to liquefy the starch followed by glycoamylase, which results in the hydrolysis of the starch to glucose (Glazer & Nikaido, 2007).

1.5.3 Lignocellulose

As mentioned before, lignocellulose is made of three kinds of polymers: cellulose, hemicellulose and lignin (Glazer & Nikaido, 2007). These components form the structure of so called microfibrils, which are organized into macrofibrils that contributes to the structural stability of the plant cell wall (Rubin, 2008). The structure of lignocellulose and its major components are shown on figure 6.

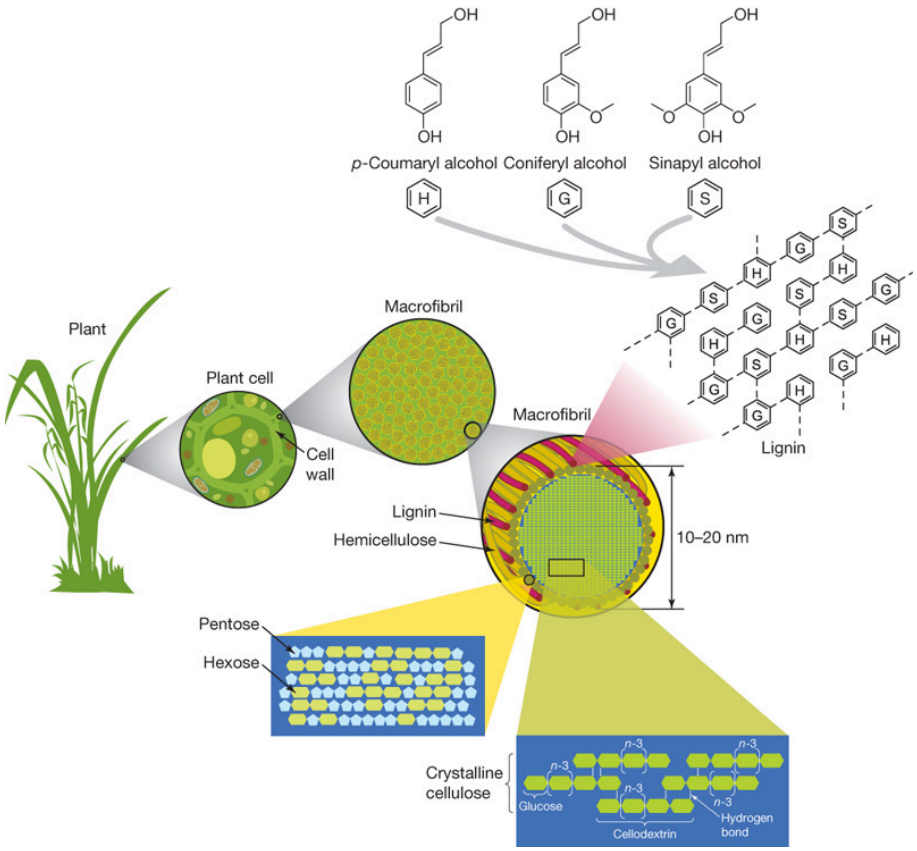

Figure 6. Structure of lignocellulose (Rubin, 2008).

The quantity of each component in lignocellulose is different between: plant species, different plant parts and the age of the plant. Lignocellulose in grasses commonly consists of 10-20% lignin, 25-40% cellulose, and 25-50% hemicellulose (Glazer & Nikaido, 2007).

1.5.4 Cellulose

Cellulose is regarded to be the most abundant organic compound on earth. Cellulose produced by plants annually is estimated more than 10^{11} tons (Glazer & Nikaido, 2007).

"The cellulose polymer chain has flat, ribbon like structure stabilized by internal hydrogen bonds." (Glazer & Nikaido, 2007). Every polymer chain consists of thousands of glucose molecules that are linked together. Cellulose is water insoluble and has a high tensile strength. Cellulose is much more resistance to degradation than other glucose polymers, such as starch. Cellulose must be hydrolysed to glucose prior to fermentation (Demirbas, 2009; Glazer & Nikaido, 2007).

1.5.5 Hemicellulose

"The components of hemicelluloses are complex polysaccharides that are structurally homologous to cellulose because they have a backbone made up of 1,4-linked β-D-pyranosyl units." (Glazer & Nikaido, 2007). Unlike cellulose, hemicellulose contains other sugars than glucose (Demirbas, 2009). Cellulose is a linear homopolymer with little variation in structure from one species to another, however hemicellulose is highly branched. Sugars present in hemicellulose include pentoses (D-xylose, D-arabinose), hexoses (D-galactose, L-galactose, D-mannose), and deoxyhexoses (L-rhamnose), and uronic acids (D-glucoronic acid) (Glazer & Nikaido, 2007). A study on timothy for ethanol production (experiment carried out in Canada) showed hemicellulose content of 23% of DM, when harvested in July at mature stage (Alvo & Belkacemi, 1997).

1.5.6 Lignin

Lignin is found in cell walls of higher plants, ferns and club mosses, mostly in the vascular tissues. Lignin increases the strength of woody tissues by lignification that gives the plants structural support and is the reason why trees can grow tens of meters upright. Lignification is a process described when lignin molecules fill up the spaces between the preformed cellulose fibrils and hemicellulose chains of the cell wall (Glazer & Nikaido, 2007). Lignin is a complex aromatic macromolecule formed by radical polymerization of phenyl-propane alcohols (*p*-coumarilic, coniferilic, and synapilic alcohols) (Milagres et al., 2011) and with high molecular weight (Demirbas, 2009).

1.6 Pre-treatment of Lignocellulosic Biomass

Prior to fermentation, lignocellulosic biomass requires pre-treatment to separate the three main components: cellulose, hemicellulose, and lignin in order to increase the yield of fermentable sugars. Pre-treatment alters the physical structure of the biomass and increases the surface area, making it more accessible for enzymes to convert polymeric carbohydrates into sugars. Various methods can be used for different types of biomass but the main aim of pre-treatment is to minimize energy cost while maximizing sugar release. Some methods can be efficient and economical for one type of biomass but are not suited for other types, there is no one pre-treatment that suits all types (Brodeur et al., 2011; Scully & Orlygsson, 2014). Schematic illustration of pre-treatment of lignocellulosic biomass is shown in figure 7.

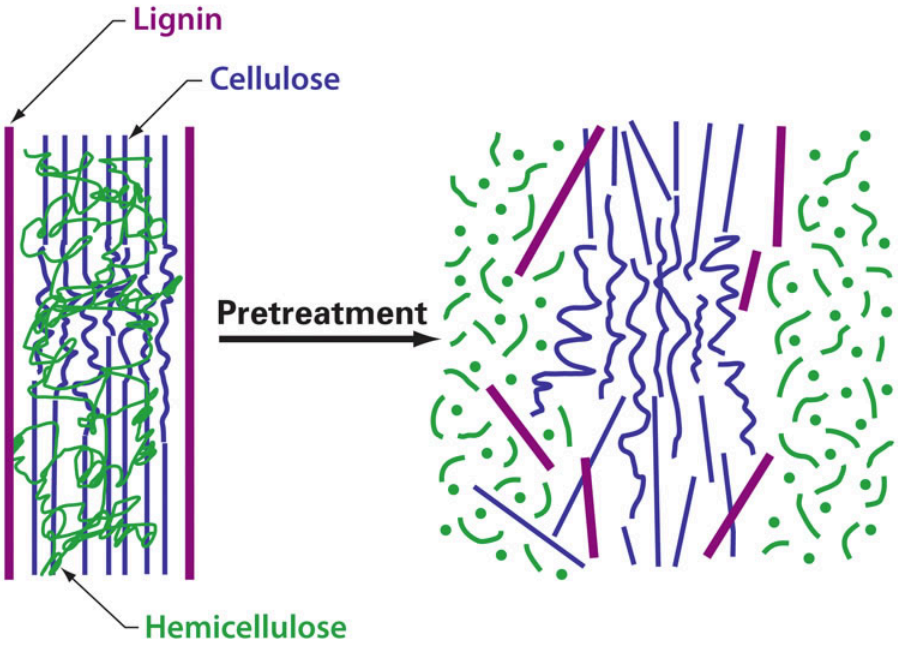

Figure 7. Schematic illustration of pre-treatment of lignocellulosic biomass. Different methods removes hemicellulose and lignin from this matrix before hydrolysis (US DOE, 2007a).

Pre-treatment methods are extremely costly processes step in biofuel production, which negatively influences its industrial feasibility and is a major obstacle for large-scale bioethanol production (Scully & Orlygsson, 2014). The high cost of the pre-treatment is thought to be the main reason why biofuel production from lignocellulosic biomass has not yet achieved a competitive stance versus traditional transport fuels (Menon & Rao, 2012).

An effective pre-treatment of biomass must meet certain requirements to be regarded a feasible choice, e.g. it has to improve the formation of sugars, minimize the degradation or loss of carbohydrates, avoid the formation of various by-products that have inhibitory effect to the fermentation process, and the pre-treatment must be cost-effective (Sun & Cheng, 2002). Pre-treatment technologies are often sorted into physical, chemical, physicochemical, and biological (Brodeur et al., 2011).

1.6.1 Physical Pre-treatment

Physical pre-treatment is required for most types of lignocellulosic biomass to reduce size and crystallinity, mechanically performed, often by milling or grinding. Reduction in biomass particle size improves hydrolysis results and mass transfers characteristics (Brodeur et al., 2011; Menon & Rao, 2012).

The main disadvantage of physical pre-treatment methods is high energy cost which depends on the final particle size. In some cases the energy cost is higher than the theoretical energy content available in the biomass. This method is very expensive and is unlikely to be used in a large-scale process (Brodeur et al., 2011).

1.6.2 Chemical Pre-treatment

Chemical pre-treatments of cellulosic materials were originally developed to use in the paper industry to produce high quality products. Chemical pre-treatment improves biodegradability of cellulose by removing lignin and/or hemicellulose and by reducing biomass crystallinity. There are many different chemical pre-treatments used including the use of acid, alkali, organic acids, pH-controlled liquid hot water, and ionic liquids (Menon & Rao, 2012).

Acid Pre-treatment

Acid pre-treatment involves the use of concentrated and diluted acids to break the structure of the lignocellulosic biomass. Diluted sulphuric acid (H_2SO_4) is the most commonly used acid for this method and can be used for many types of different biomass: e.g. switchgrass, corn-stover, spruce (softwood), and poplar. Originally, dilute sulphuric acid was used to produce furfur aldehyde by hydrolysing the hemicellulose to simple sugars that continued to convert into furfuraldehyde. Other acids have also been used for pre-treatment of biomass e.g. hydrochloric acid (HCl), phosphoric acid (H_3PO_4), and nitric acid (HNO_3) (Brodeur et al., 2011; Menon & Rao, 2012).

Acid pre-treatments are often used to remove hemicellulose and combined with other methods in overall biomass fractioning (Menon & Rao, 2012). A good advantage of acid treatment is that in some cases an enzymatic hydrolysis is not required because the acid itself hydrolyses the biomass to fermentable sugars. Both hemicellulose and lignin are solubilized with minimal degradation, and hemicellulose is converted to sugars with acid pre-treatment (Brodeur et al., 2011).

Main disadvantages of using acid pre-treatment are the production of inhibitory compounds like 5-hydroxymethyl-2-furfuraldehyde (5-HMF) and furfuraldehyde that reduce both the effectiveness of the pre-treatment and further process (Brodeur et al., 2011). Also, most acids are corrosive, toxic and hazardous, therefore adequate material for

the reactor is needed. In addition, it is important to recover the concentrated acid to make the process economically feasible (Sun & Cheng, 2002).

Alkaline Pre-treatment

Alkaline pre-treatment of lignocellulosic biomass is performed with the use of bases, such as sodium, potassium, calcium and ammonium hydroxide. The alkali causes the degradation of ester and glycosidic side chains, which leads to structural alteration of lignin, cellulose swelling, partial decrystallization of cellulose and partial solvation of hemicellulose. The structural alterations of the biomass results in better accessibility of enzymes to both cellulose and hemicellulose. This method has worked best on biomass like corn stover, switchgrass, bagasse, wheat- and rice straw (Brodeur et al., 2011).

1.6.3 Physico-chemical Pre-treatment

Physico-chemical pre-treatment is method combined from chemical and physical process (Harmsen et al., 2010). There are many different physico-chemical pre-treatments available including steam explosion, liquid hot water, ammonia fiber explosion, wet oxidation, ozonolysis pre-treatment, acid hydrolysis, and organosolv processes (Taherzadeh & Karimi, 2008). A short overview is given on the first three mentioned methods.

Steam Explosion

Steam explosion is most commonly used of physico-chemical methods for pre-treatment of lignocellulosic biomass (Sun & Cheng, 2002). This method removes most of the hemicellulose and therefore improves enzymatic digestion. The process involves injecting high-pressure saturated steam into a batch or continuous reactor filled with biomass causing a rapid increase in temperature (up to 160-260°C). Then, the pressure is rapidly reduced causing explosive decompression of the biomass. The hemicellulose degrades and the lignin structure is disrupted (Taherzadeh & Karimi, 2008). The outcome of this method depends on the residence time, temperature, particle size, and moisture content of the biomass (Sun & Cheng, 2002). Some effort has been done to try to improve the results of steam explosion by adding acid or alkali to the treatment (Harmsen et al., 2010).

Liquid Hot Water

This treatment is performed with the use of water at high temperature (160-240°C) and high pressure that maintains to promote the disintegration and separation of the lignocellulosic matrix (Brodeur et al., 2011). Water under high pressure can penetrate into the biomass, hydrate the cellulose, and remove the hemicellulose. Part of the lignin fraction is also removed. The main advantage of this method is that there is no addition of chemicals and therefore no requirement for corrosion resistance materials for hydrolysis reactors (Taherzadeh & Karimi, 2008).

Ammonia Fiber Explosion (AFEX)

AFEX is categorised with alkaline physico-chemical pre-treatment methods. The biomass is exposed to liquid ammonia at relatively high temperature (e.g. 90-100°C) for a period of time (e.g. 30 minutes) followed by reduction of pressure (Taherzadeh & Karimi, 2008). This method is similar to steam explosion but the temperature used is lower and that results in less energy input and lower overall cost (Brodeur et al., 2011).

AFEX is more effective on biomass where the lignin content is relatively low. This treatment does not solubilize hemicellulose like dilute-acid treatment for example (Taherzadeh & Karimi, 2008). The saturation of the ammonia and biomass mixture is saturated for a period of time and then reduction in pressure leads to rapid expansion of the ammonia gas, which causes swelling of the biomass. The swelling leads to a break in the structure of the lignin, hemicellulose, and cellulose. This results in more accessible surface area for enzymatic digestion (Brodeur et al., 2011). The ammonia must be recovered after the treatment because of cost and environmental effect (Taherzadeh & Karimi, 2008).

1.6.4 Biological Pre-treatment

Biological pre-treatment is usually done with the use of microorganisms, including white-, brown-, soft-rot fungi and bacteria to alter the structure of the lignocellulosic biomass to make it more accessible for enzyme digestion (Menon & Rao, 2012; Sun & Cheng, 2002). The goal is to degrade lignin and hemicellulose without degrading the cellulose. When fungi are used for pre-treatment, the lignin degradation can be performed by lignin degrading enzymes produced by the fungi (Brodeur et al., 2011).

The main advantages of biological pre-treatment are mild conditions and no chemical requirement. However, this method is slow compared to other options, the rate of hydrolysis are low and it requires both careful control of growth conditions and a lot of space to perform this type of treatment (Menon & Rao, 2012; Sun & Cheng, 2002). The main problem with biological treatment is that most lignolytic microorganisms solubilize not just the lignin part but also the hemicellulose and cellulose. This is regarded a major technical challenge and is therefore less attractive commercially (Menon & Rao, 2012). Current research is focused on combining biological pre-treatment with other methods (Brodeur et al., 2011).

1.6.5 Enzymatic Hydrolysis

Enzymatic hydrolysis is one of the most effective methods to convert cellulosic materials to simple sugars (Talebnia et al., 2010). On the other hand, the use of commercial enzymes for this conversion is regarded to be the most expansive step in the production of bioethanol from lignocellulosic biomass (Scully & Orlygsson, 2014). Saccharification is a critical step for the ethanol production, when complex carbohydrates are converted to simple monomers (Sarkar et al., 2012).

There are two main types of processes to hydrolyse the cellulosic biomass after the pre-treatment. The most commonly used methods are chemical or enzymatic hydrolysis (Demirbas, 2009). The main advantages of enzymatic hydrolysis are low toxicity, low utility cost, and low corrosion when compared to chemical hydrolysis. Also, no inhibitory compounds are formed during enzymatic hydrolysis (Sarkar et al., 2012).

Both the pre-treatment method and the conditions during the hydrolysis have great effect on the efficiency of the enzymatic hydrolysis, like temperature and pH. Many enzymes show optimum activity at temperature and pH between 45-55°C and 4-5, respectively (Talebnia et al., 2010).

There are three main groups of enzymes that are important for hydrolysing cellulose to glucose: endo-glucanase, exo-glucanase, and ß-glucosidase. These enzymes have different effect but their action is synergistic. Endo-glucanase attacks the low crystallinity in the cellulose fiber and creates free chain-ends. Exo-glucanase continues to degrade the

molecule with the removal of cellobiose units from the free chain-ends. ß-glucosidase then cleaves the units to glucose (Talebnia et al., 2010).

Another possible method is to use celluolytic microorganisms to degrade cellulose and hemicellulose (biological pre-treatment). There are some well-known cellulose degraders that can alter the structure of lignocellulosic biomass so it becomes more accessible to enzymes. Degradation of cellulose requires cellulases, that can be produced by organisms such as *Clostridium thermocellum*, or by fungi like *Trichoderma reesei* or *Aspergillus niger* (Scully & Orlygsson, 2014). Most studies for commercial cellulase production have focused on fungi because most of the suitable bacteria are anaerobes with very low growth rates (Talebnia et al., 2010).

1.6.6 Inhibitory Effects of Pre-treatment

The pre-treatment of lignocellulosic biomass does not only have positive effects on the fermentation process (Palmqvist & Hahn-Hagerdal, 2000). Several inhibitory compounds are often formed during the process. For example, furfuraldehyde, 5-HMF, and aromatic compounds derived from lignin such as *p*-coumaryl alcohol, coniferyl alcohol, sinapyl alcohol and related derivatives (Scully & Orlygsson, 2014). Also, weak acids, mostly acetic, formic, and levulinic acids (Alvira et al., 2010). These compounds may have negative effect on the microbial growth and fermentation process, even in relatively low concentrations (Scully & Orlygsson, 2014). The effect of inhibitory compounds will often result in lower ethanol and hydrogen productivity and yields, respectively (Chang & Yao, 2011; Palmqvist & Hahn-Hagerdal, 2000). The levels of inhibitory compounds formed during the pre-treatment of the biomass are based on various factors like: type of biomass, solid contents and pre-treatment conditions (temperature, pH, concentration of chemicals) (Chang & Yao, 2011).

These inhibitory compounds are problematic for the ethanol production and therefore several different strategies have been suggested to minimize the formation of inhibitors during hydrolysis including detoxification of the hydrolysates (HLs) prior to fermentation, development of inhibitor tolerant microbes, or convert inhibitors to non-inhibiting compounds (Taherzadeh et al., 2000).

1.6.7 Fermentation

"In microbiology, fermentation is defined as a metabolic process leading to the generation of ATP and in which degradation products of organic compounds serve as hydrogen donors as well as hydrogen acceptors. Oxygen is not a reactant in fermentation processes." (Glazer & Nikaido, 2007).

"The theoretical yields of ethanol from 1 mol of hexose and pentose are 2.0 mol and 1.66 mol, respectively":

$$1.00\ C_6H_{12}O_6 \rightarrow 2.00\ C_2H_5OH + 2.00\ CO_2$$

$$1.00\ C_5H_{12}O6 \rightarrow 1.66\ C_2H_5OH + 1.66\ CO_2$$

However, these yields are never obtained because a part of the substrate is converted to biomass or to other end products. The formation of different end products depend both on organisms used and environmental factors that affect the process (Scully & Orlygsson, 2014).

Commonly, thermophiles use glycolysis (the Embden Meyerhof pathway) (Taylor et al., 2009), which is the major pathway of glucose metabolism and adenosine triphosphate (ATP) synthesis in anaerobic organisms and first stage of glucose metabolism in aerobic organisms. This pathway leads to the yield of two moles of pyruvate, 2 NADHs, and net gain of 2 ATP for one mole of glucose utilized (Staley et al., 2007). A simplified scheme of glucose degradation to various end products by strictly anaerobic bacteria is shown in figure 8.

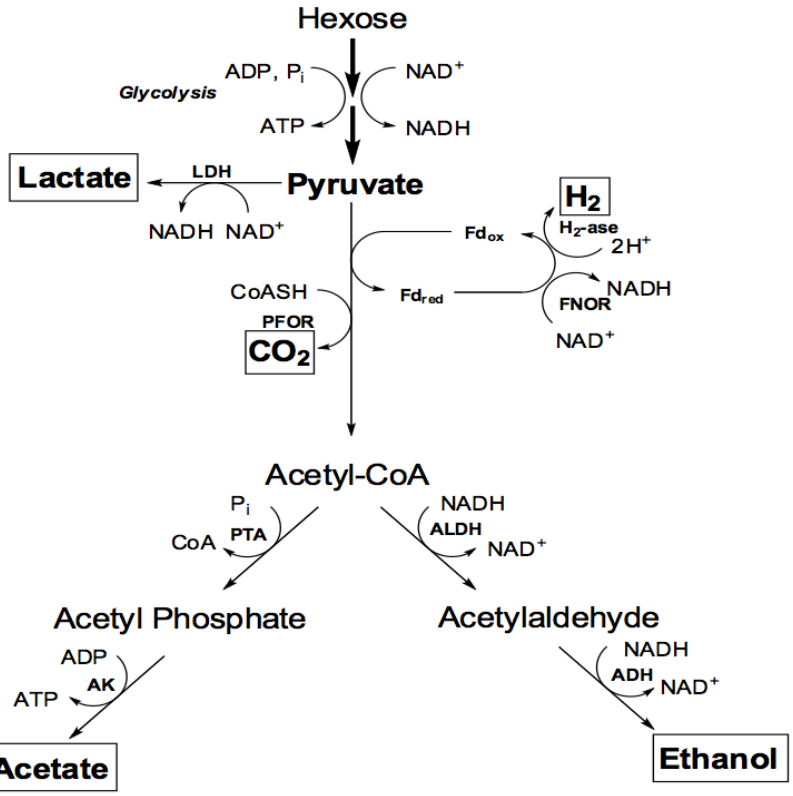

Figure 8. Simplified scheme of glucose degradation to various end products by strictly anaerobic bacteria. Enzyme abbreviations: ALDH—acetaldehyde dehydrogenase; ADH—alcohol dehydrogenase; AK—acetate kinase; FNOR—ferredoxin oxidoreductase; H_2-ase—hydrogenase; LDH—lactate dehydrogenase; PFOR—pyruvate ferredoxin oxidoreductase; and PTA—phosphotransacetylase (Scully & Orlygsson, 2014).

After glycolysis under anaerobic conditions, pyruvate can be converted to several end products like organic acids, alcohols, CO_2 and hydrogen. Anaerobic yeast decarboxylate pyruvate to acetaldehyde and further to ethanol (Staley et al., 2007).

Alternative pathway, Entner-Doudoroff pathway yields one ATP with the formation of two moles of pyruvate. Additionally one mole NADPH and one mole NADH is also formed per mole glucose (Staley et al., 2007).

Under aerobic conditions, organisms oxidize glucose in process called respiration. This pathway can generate 38 ATP from one mole glucose. Other end products formed during this process are primarily CO_2 and water (Jurtshuk, 1996).

1.7 Bioethanol Processes from Lignocellulosic Biomass

Converting lignocellulosic biomass into ethanol is a very complex process. After suitable pre-treatment, there are four main process methods (figure 9), with the use of various organisms: Separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF), and consolidated bioprocessing (CBP) (Chang & Yao, 2011). Each process is discussed below.

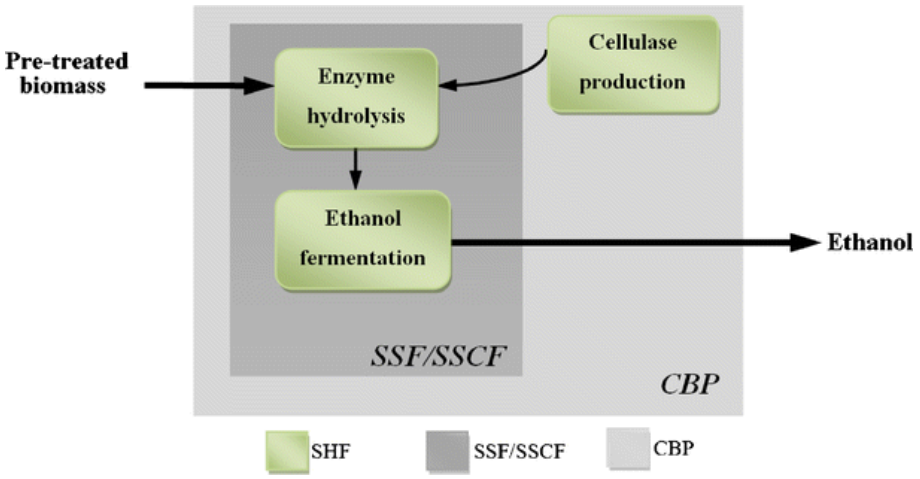

Figure 9. A simplified scheme for different processes of lignocellulosic biomass to ethanol. Three steps for SHF, cellulase production, enzyme hydrolysis and ethanol production. SSF and SSCF combine the enzyme hydrolysis and fermentation. The CBP method combines all processes (Chang & Yao, 2011).

SHF

Separate hydrolysis and fermentation (SHF) is a process where the biomass hydrolysis, and fermentation is performed in separate steps. That allows each step to be carried out at its optimal environmental conditions (temperature and pH) (Chang & Yao, 2011). The main disadvantage with this method is that the glucose produced during biomass saccharification has inhibitory effect on the cellulase activity, especially β-glucosidase. This results in much lower reaction rate (Alfani et al., 2000), and limits the process efficiency (Chang & Yao, 2011).

SSF

Simultaneous saccharification and fermentation (SSF) is a process in which the cellulose is degraded and fermented at the same time (Brodeur et al., 2011). There is a potential for high production rates by using this method because the removal of inhibitors from the

reaction medium is continuous, which keeps the depression of enzyme activity at minimum (Alfani et al., 2000).

SSCF

Simultaneous saccharification and co-fermentation (SSCF) is a convenient method for ethanol production from xylose-rich lignocellulosic biomass (Menon & Rao, 2012). SSF and SSCF are similar processes except SSCF also includes the pentose fermentation, which is important for higher efficiency of ethanol production. Both these processes result in higher ethanol yield and productivity when compared to SHF process (Chang & Yao, 2011).

CBP

Consolidated bioprocessing (CBP) combines the three steps of bioconversion of biomass into a single step: cellulase production, enzymatic hydrolysis, and fermentation. The main advantages of this method are potential for low cost and higher efficiency, when compared to other processes. A microbial culture used for this method must combine properties for both substrate utilization and product formation (Menon & Rao, 2012).

1.8 Ethanol Producing Organisms

As mentioned earlier, there is a long history of first generation ethanol production. However, ethanol production from more complex biomass is more recent subject. Wiegel et al. (1983) reported their data on promising ethanol production from hemicellulose (birch and beechwood sources) by the use of *Thermoanaerobacter ethanolicus* (strain JW200), with yields of 0.81 mol ethanol/mol xylose. *Clostridium thermocellum* is another promising thermophilic bacteria and has shown good ethanol yields from Whatman paper, or up to 8.0 mM ethanol/g substrate (Rani et al., 1998). *Zymomonas mobilis* is an interesting bacterium that first attained attention in 1912 as contributors to "cider sickness" or spoilage of fermented apple juice. *Z. mobilis* produces ethanol rapidly and with an ethanol yield up to 97% of theoretical yield from glucose. However, this bacterium uses only glucose, fructose, and sucrose and the yield from the latter two substrates is lower than from glucose (Glazer & Nikaido, 2007). Recently isolated *Thermoanaerobacter* strain J1, isolated from a hot spring in Iceland has showed with a broad substrate spectrum to degrade various sugars and starch. The yield has been up to 1.70 mol ethanol/mol glucose

and 1.25 mol ethanol/mol xylose although the strain does not degrade cellulosic substrates (Jessen & Orlygsson, 2012). The most commonly used yeast for alcohol fermentation is *S. cerevisiae,* which exhibits fast sugar consumption and gives high ethanol yields (Becker & Boles, 2003), more than 1.9 mol ethanol/mol hexose (Wiegel, 1980). The thermotolerant yeast *Kluyveromyces marxianus* is another interesting ethanologen. *K. marxianus* has a broad substrate spectra and showed some promising results as an ethanol producer (Limtong et al., 2007), especially from whey and some strains can degrade lactose (Zafar & Owais, 2006), in contrast to *S. cerevisae* (Sreekrishna & Dickson, 1985). Examples of ethanol production by selected organisms are shown in table 1.

Table 1. Examples of ethanol production by selected organisms. Cultivation was in batch and ethanol yields are given in mM/g substrate degraded or mM/g of initial concentration (*). Substrate concentration and incubation temperature are also shown.

Organism	Substrate	Substr. conc. (g/L)	Ethanol yield (mM/g)	Temp. (°C)	Reference
C. thermocellum	Whatman paper	8	7.2-8.0	60	(Rani et al., 1998)
T. ethanolicus	Wood HLs	8	3.30-4.50	70	(Wiegel et al., 1983)
Thermoanaerobacter strain J1	Whatman paper	4.5	7.5	65	(Jessen & Orlygsson 2012)
Z. mobilis 8b6	Corn stover	N/A	9.1*	30	(Hahn-Hagerdal et al., 2006; Mohagheghi et al., 2004)
K. marxianus	Barley straw	3.9	4.3	45	(Boyle et al., 1997)
S. cerevisiae 424A(LNH-ST)	Corn stover	125	10.6	30	(Sedlak & Ho, 2004)

Abbreviations: Not available (N/A).

1.9 Biomass Production in Iceland

There has been increased discussion of possibilities to produce fuel from biomass in Iceland. There are some ideas that domestic fuel production could replace some part of imported fossil fuel (H. Bjornsson, 2006; Sveinsson & Hermannsson, 2010). This would be an important factor considering the sustainability of energy use and production. Biomass could possibly be a good opportunity for Icelandic agriculture in addition to other production.

The short and rather cold summer is not favourable for many of the most commercially grown food and fuel crops such as corn, wheat, and sugar beets. Timothy is the most

important forage grass in Icelandic agriculture and therefore it is interesting to see if it is possible to use timothy as feedstock for bioethanol production.

1.9.1 Icelandic Climate

Iceland is located in the North Atlantic Ocean. The Icelandic climate during winter is surprisingly mild and the summers are rather cool, given its location and degree of latitude (extends between the latitudes 63.2°N and 66.3°N) (Helgadottir et al., 2013). This is because of the Gulf Stream effect. The country is located on the broad boundary between two air currents, one of polar origin and the other one of tropical origin. Two ocean currents have also major effect on Icelandic climate: the Gulf Stream, from near the Equator and the East Greenland Current. Temperatures do not vary much throughout the country nor between seasons. The mean annual temperature for Reykjavík is 4°C. The mean January temperature is −0.5°C, and the mean July temperature is 11°C. Annual precipitation ranges from 410 mm in some high northern plateaus to more than 4,100 mm in some of the southern slopes of ice-capped mountains. Average annual precipitation in the south is about 2,000 mm (Iceland, 2015).

It is more likely that climate and weather conditions sets limits for farming in Iceland, rather than soil type (Snaebjornsson et al., 2010). Even though arable land is available, in vast areas with suitable soil type, the climate and weather must be suitable for the vegetation growth. Hermannsson (2001) made attempt to categorize Icelandic farmland into three growing zones. This was mostly done by comparing summer temperature but also considered soil frost depth and soil type. Hermannsson noted that this estimation was not very accurate and there is uncertainty for farmland that is between zone categories or close to boundaries of a zone. Usable heat for plant growth is dependent on two variables: length of growing season and the mean temperature during that time. The growing season in Iceland is 130 days, starting May 7th on average, when mean temperature is exceeding 5°C. Usually at that time, grasses turn green and tillage can be performed. But there are some exceptions, this can happen earlier in some of the coastal regions in the south. The growing season ends on average September 15th when temperature is below 7°C. Hermannsson defined three zones as shown in table 2.

Table 2. Growing cultivation zones in Iceland (Hermannsson, 2001).

Zone	Average temperature in summer (°C)	Growing day degrees (GDD)	Elevation, meters above sea level (MASL)	Area location
1	10	1300	100	South part and the upcountry and best areas of the west-, northeast- and east part
2	9	1170	100-200	Other parts of the lowland except for the coastal region in northwest and east part
3	8	1040	100-200	Coastal region in northwest and east part

As shown in table 2, the possibility to grow crops in Iceland is limited by the total of growing day degrees (GDD). It is important to choose crops and varieties that fit the local conditions because crops that need the mean number of GDD to grow, would only give harvest every other year because of variation between summer seasons. If 100 GDD are added to the crop requirement it should be possible to get 4 harvests out of every 5 growing seasons (Hermannsson, 2001). Heat requirements of several common crops are listed in table 3.

Table 3. GDD requirements for several common crops (Hermannsson, 2001).

Crop type	GDD
Forage crop, 1 harvest	500
Barley, early maturity	1200
Barley, mid maturity	1350
Oats, early maturity	1400
Wheat, early maturity	1600
Turnip	1100
Swede/Yellow turnip, ready to sell	1200
Swede/Yellow turnip, fully grown	1400
Potatoes, pre-sprouted	1200

As shown in table 3, it is clear that most of these crops require total number of day degrees that are around the boundary of being possible to grow and harvest in Iceland, with certain guarantee of acceptable result.

1.9.2 Timothy

Timothy (*Phleum pratense* L.) is a cool-season forage grass, adapted to temperate, moist environments. Timothy does not thrive in draughty climate. Timothy is widely grown, it is a popular forage grass in the cool, moist regions of the US, Canada and Europe. It is also grown in northern Europe, temperate regions of South America, Australia and Japan. Timothy is very important forage in areas where winters are harsh. Development of the plant is related to GDD. In Atlantic Canada, timothy cultivars need 350-450 GDD (>5°C) to reach early heading stage. Moreover, diseases or insects pest are normally not a serious problem or limitation for timothy growth (Mcelroy & Kunelius, 1995).

Importance in Icelandic Agriculture

"Timothy (*Phleum pratense* L.) is the most important forage grass in Icelandic agriculture" (Helgadottir & Sveinsson, 2006). Timothy has good yield ability, feed quality, palatability and persistence (Helgadottir & Sveinsson, 2006; Sveinsson, 2001). Timothy is a naturalized species in Iceland, usually distributed in cultivated pastures and near residential areas (Kristinsson, 2004). Timothy is highly favoured among Icelandic farmers and it was estimated according to seed imports in the period of 1995 to 2006 that timothy made up to 75-85% of the total area sown with forage grasses (Helgadottir & Sveinsson, 2006).

Plant Description

Timothy is a perennial bunchgrass that can grow up to about 1 meter in height. It forms an open sod and is not an aggressive plant and therefore associates well with other species in mixtures. It is a long-day plant and does not require cold period for flower induction. The leaf blades are flat and often twisted. They are hairless aside from sparse cilia on the blade collar. The panicles are compact, cylindrical and spike-like, usually 5-10 cm long and 6-10 mm thick (Mcelroy & Kunelius, 1995).

The root system of timothy has no rhizomes, it is fibrous but very shallow, up to 80% of the root mass has been found in the top 5 cm of the soil. Regrowth is usually poor under dry conditions, partly a result of a shallow root system (Mcelroy & Kunelius, 1995).

Importance and Use

Timothy is a popular choice where it is adapted. It produces good yields of high quality forage when harvested at the early heading stage. It is commonly grown to use as conserved feed, as hay or silage. It is widely grown in mixtures with a legume, alfalfa (*Medicago sativa* L.), red clover (*Trifolium pratense* L.) or birdsfoot trefoil (*Lotus corniculatus* L.). Timothy is often grown in pure stands in areas where winter conditions or drainage are not favourable for legumes (Mcelroy & Kunelius, 1995). In Iceland, timothy is commonly grown in mixture with meadow-grass (*Poa pratensis* L.) (Thorvaldsson, 1998, 1999).

Harvest Time and Persistence

Studies in Iceland have shown that harvesting timothy in early summer (early growth stage) results in less persistence (Hermannsson & Helgadottir, 1991). Timothy is sensitive for frequent cutting during stem elongation. This is a critical period for the plant persistence and will likely result in rapid decline in plant cover (Mcelroy & Kunelius, 1995). If timothy is first harvested in mid-summer or later it is possible to maintain good cover for a long time (Hermannsson & Helgadottir, 1991). There is little known on the effect of the second harvest time on the timothy cover (Sveinsson, 2001).

1.9.3 Different Harvest Time of Timothy

Timothy is most productive in spring and early summer. Dry matter (DM) yields increase to anthesis and maximum yield can be achieved at that growth stage. On the other hand, forage quality drops rapidly as the plant mature. As said before, timothy is sensitive for cutting during stem elongation. Therefore, a compromise between yield, quality and persistence must be made when choosing a harvesting time. Harvesting timothy at early- to mid-heading stage of development is thought to be a good compromise and should result in quality of forage and moderate persistence (Mcelroy & Kunelius, 1995).

Optimal harvest time depends on the planned use of the harvest. When forages are harvested as animal feed, the harvest time has a crucial effect on the dry matter digestibility (DMD) of the forages. Forages DMD is highest in spring but declines as the plant mature during the summer (Thorvaldsson, 2003). The DMD of forages depends on the cellular content and the digestibility of the cell wall. Cellulose in young grasses is

almost completely digestible for ruminants, but as the plant mature the DMD will rapidly decline (Thorvaldsson, 2006), as shown in figure 10.

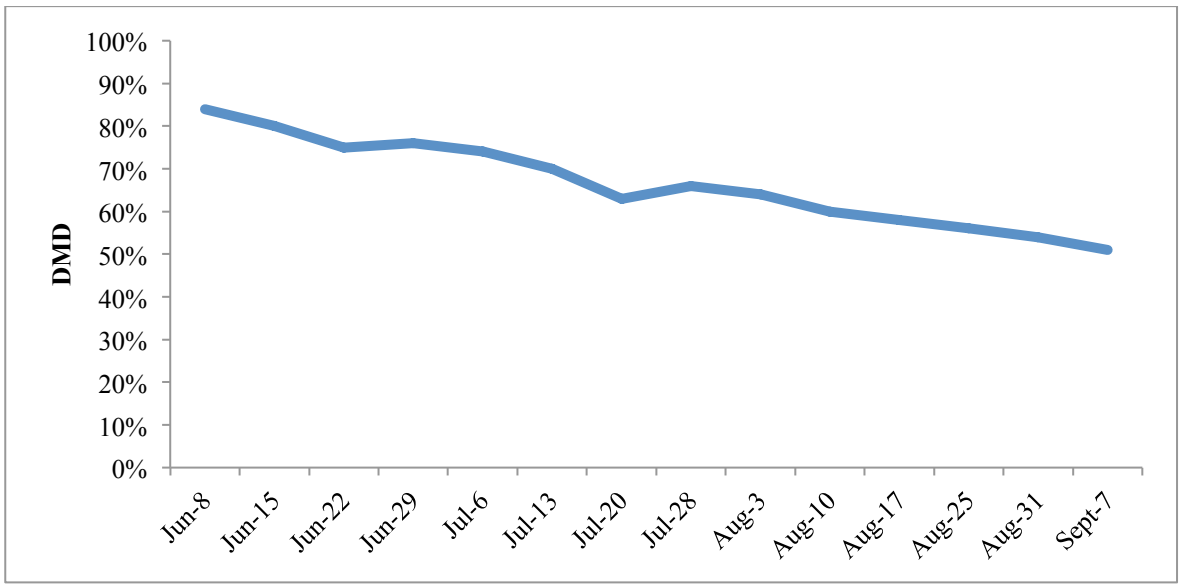

Figure 10. Declining DMD of timothy as it mature. Results from 14 harvest dates during the summer of 1966 (Olafsson, 1979).

The DMD of the timothy declines more rapidly with increased maturity when compared to many other forage species. An exception is meadow foxtail (*Alopecurus pratensis* L.), which has similar decline in DMD (I. Bjornsson, 2000). Early harvested (primary cut) of timothy is an excellence feed and with very good DMD (Helgadottir et al., 2013; Helgadottir & Sveinsson, 2006; Sveinsson & Bjarnadottir, 2006). But if the timothy is harvested late summer and only one time, it will not have these good qualities (Sveinsson, 2001). "Therefore, farmers have been advised to cut timothy no sooner or later than at mid-heading to secure persistence, high DM yield and feed quality since timothy regrowth is usually poor and unreliable." (Sveinsson & Bjarnadottir, 2006).

Timothy Yield

Common yield of timothy in Iceland is about 8 t DM/ha when harvested in early August. In 1979 and 1980, timothy was sampled weakly throughout the summer for yield comparison, at Korpa experimental station in SW Iceland. The summer of 1979 was cold and short but the maximum yield of 9.2 t DM/ha was obtained on August 28th. The summer in 1980 was more favourable, early and warm and maximum yield of 10.5 t DM/ha was obtained on August 12th. These values can be regarded as potential maximum

yield of timothy in Iceland. This gives growth rate of about 140 kg DM/ha/day over more than 50-day period (H. Bjornsson, 1987; H. Bjornsson et al., 2004). An example of timothy yield depending on harvest time is shown on figure 11. The data is from a four-year experiment on mowing height (values are from plots mowed at low heights) at Möðruvellir in Hörgárdalur (Sveinsson, 2003).

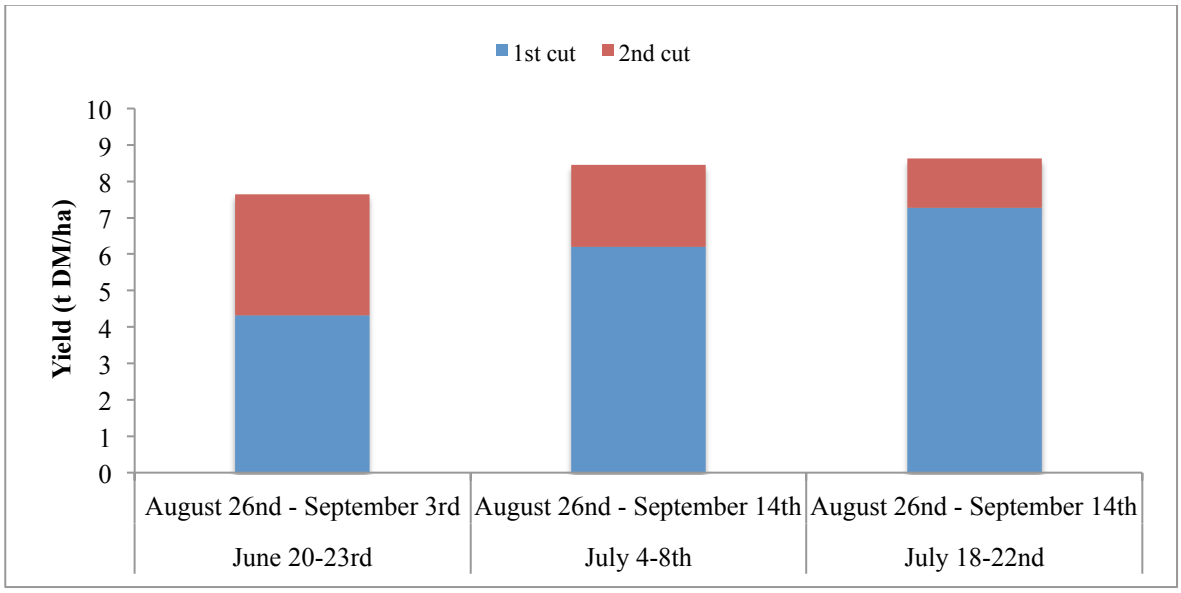

Figure 11. The effect of harvest date on timothy yield. Mean of four harvest seasons: 1999-2002 at Möðruvellir experimental farm in Hörgárdalur North Iceland (modified from Sveinsson, 2003).

Timothy for Biomass Production

There has been some discussion on using timothy as feedstock for ethanol production (H. Bjornsson, 2006; H. Bjornsson et al., 2004; Sveinsson & Hermannsson, 2010). Perennial grasses are an interesting source for biomass production, because of high yield and relatively low production cost. There have been some studies on fuel production from grasses, both biogas and bioethanol production (Sveinsson & Hermannsson, 2010). Researches on biogas production from grasses have shown great variation both between grass types and harvest times (Mahnert et al., 2005; Seppala et al., 2009).

As a part of their project, *Feasibility study of green biomass procurement*, H. Bjornsson et al. (2004) carried out experiment to examine the effect of five different unconventional harvest times of timothy with focus on biomass production. Both yield and nutrient value was measured. The harvest dates were from the 18th of August 2003 to 24th of May 2004.

The yield resulted highest in plots harvested in late summer and fall, (H. Bjornsson et al., 2004; Sveinsson & Hermannsson, 2010), as shown on figure 12.

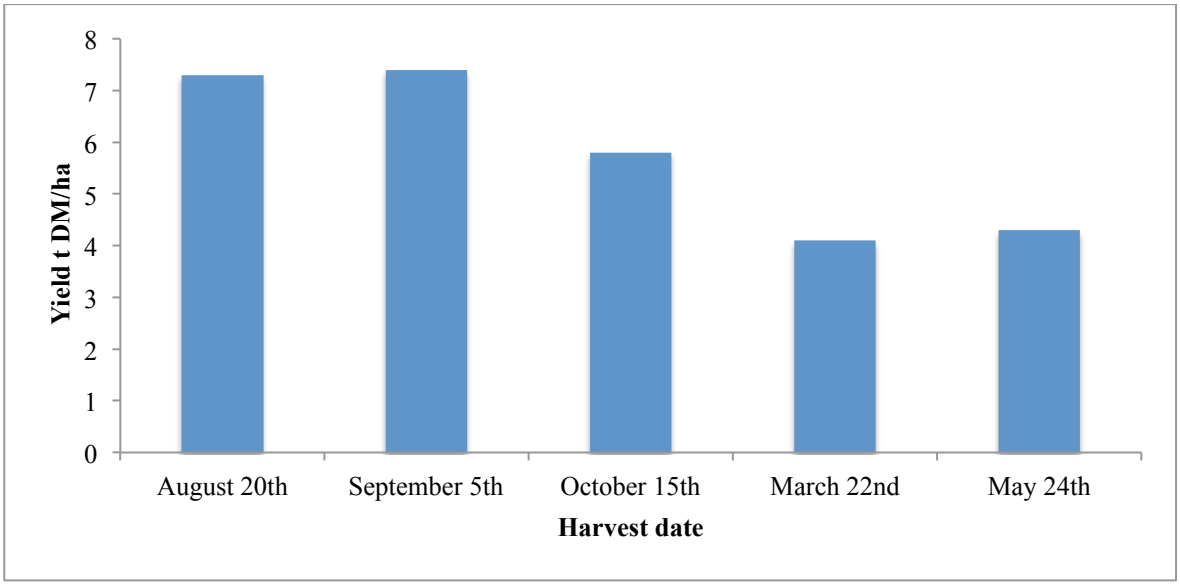

Figure 12. Effect of different, unconventional harvest time of timothy on primary DM yield. Harvested in fall and winter 2003-2004 (modified from H. Bjornsson et al., 2004).

The main reason for lower yield in late winter than in the fall is because of weathering during autumn and winter. Cell and cell wall contents were also examined. There was no significant difference between samples from August 20th and September 5th. In autumn and winter, the water-soluble cell materials decreased and cell wall material (Nutrient Detergent Fibres (NDF)) increased (from 61-77% of the DM). Some minor decline in hemicellulose was detected, relative to NDF (H. Bjornsson et al., 2004).

1.9.4 Previous Studies of using Timothy for Ethanol Production

Recent experiments carried out at the University of Akureyri showed interesting results on ethanol production from timothy by using the thermophilic bacterium, e.g. *Thermoanaerobacter* strain J1 and *Thermoanaerobacter* strain B2 (Jessen & Orlygsson, 2012; Orlygsson, 2013; Sveinsdottir et al., 2009). The timothy samples were harvested June 26th. 2012 (Jessen, 2013) at mid-heading stage at Möðruvellir Experimental Station in Hörgárdalur, near Akureyri (Þóroddur Sveinsson, personal communication, February 17th, 2015). *Thermoanaerobacter* strain B2 produced 4.2 mM of ethanol/g DM of timothy (Orlygsson, 2013). This is about 0.24 litres ethanol/kg DM or 1200-1700 litres ethanol/hectare, depending on DM yield (5-7 t/ha).

As a part of his master thesis, Jessen (2013) compared different ethanol producing strains using different types of lignocellulosic biomass, including ethanol production by *Thermoanaerobacter* strain J1 on previously mentioned timothy samples from Möðruvellir. The most promising results from his experiments by this strain resulted in ethanol production of 11.7 mM from timothy HLs at concentration of 2.5 g/L, or 4.7 mM/g timothy.

Sveinsson and Hermannsson (2010) reviewed recently that likely production from lignocellulosic biomass is estimated 0.27 L ethanol/kg DM in timothy. But they noted that studies have shown very variable yield results, depending on: different biomass quality, pre-treatment method, and organisms used.

1.9.5 Effect of Harvest Time on Ethanol Production

Many studies have investigated harvest time effect on crop yield and quality and several focused on biomass production for bioenergy production. However, little information was found on the subject of comparing different harvest time on ethanol production from grasses.

The harvest time affects both yield and the crop quality and the optimal harvest time depends on the planned use of harvest. Pahkala et al. (2007) studied the effect of harvest time on overall potential of barley straw and reed canary grass (RCG) as feedstock for bioethanol production in Finland. The total yield of barley straw was 8-10 t DM/ha but the highest yield for RCG was 11 t DM/ha. Cellulose and lignin content was measured as a part of this experiment. The cellulose and lignin content of the barley straw increased until dough stage of grain but for the RCG the cellulose and lignin content was higher with increased maturity. The biomass was treated with stem explosion and hydrolysed with enzymes. Samples were fermented by yeast to see if ethanol would be produced from the material. Pahkala et al. (2007) concluded that both barley straw and RCG were found to be suitable materials for ethanol production. It could easily be pre-treated with stem explosion, hydrolysed to monosaccharides and fermented to ethanol.

Vogel et al. (2002) investigated the optimal harvest periods for switchgrass (*Panicum virgatum* L.), when grown for biomass production for bioenergy in the Midwest USA. This study was based on experiment carried out in 1994 and 1995. The first harvest was cut

from late June until late August with 7 days interval (7 harvest dates). Additional harvest (harvest 8) was performed after a killing frost and the regrowth of previously harvested plots was harvested at the same time. The results showed that the optimum biomass yield (first cut), when averaged over years, was obtained at the maturity stages between full-heading until post-flowering. The yield obtained at that maturity stages was between 10.6-12.6 t/ha (first cut), depending on year and research site. Sufficient yield may be harvested from a second cut after a killing frost.

Another study focused on the biomass yield and biofuel quality of switchgrass (*Panicum virgatum* L.) when harvested in fall or spring. The yield was less when harvested in spring but delaying harvest reduced ash and water concentration that may reduce transport costs (Adler et al., 2006).

Belkacemi et al. (1998) carried out an experiment in eastern Canada to study ethanol production from AFEX treated lignocellulosic biomass, derived from forages and agricultural residues. Forages used were; mature timothy grass (*Phleum pratense* L.), alfalfa (Medicago sativa L.) and RCG (*Phalaris arundinacea* L.), mowed in July 1995 and stored as dry baled hay. Agricultural residues used were; corn stalks (*Zea mays* subsp. *mays* L.) and barley straw (*Hordeum vulgare* L.), baled in August and October 1995, respectively. The biomass was pre-treated with ammonia fiber explosion (AFEX) and also enzymatically hydrolysed. HLs were prepared from the biomass contained 20-24 g/L of total sugar. The ethanol producing yeast *Pachysolen tannophilus* (ATCC 32691) was used for this experiment. The ethanol yield varied from 31% to 62% of theoretical yield based on initial sugars available for the fermentation or from 53% to 100% of theoretical yield based on consumed sugars, as shown in table 4.

Table 4. Ethanol production from HLs made from different lignocellulosic biomass. Samples taken after 24 hours and 45 hours (modified from Belkacemi et al., 1998).

Biomass	Sugar uptake (% of initial content)		Ethanol (g/L)		Yield[a] (% theoretical)		Yield[b] (% theoretical)		Productivity (g EtOH/L.h)	
	24h	45h	24h	45h	24h	45h	24h	45h	24h	45h
Corn stalks	46.6	48.9[c]	3.8	3.7[c]	37	37[c]	79	75[c]	0.16	0.08[c]
Barley straw	47.7	65.0	4.5	6.3	44	62	92	95	0.19	0.13
Alfalfa	37.2	54.0	3.8	5.7	38	56	100	100	0.16	0.12
Timothy	66.1	67.9[c]	4.1	4.4[c]	35	37[c]	53	55[c]	0.15	0.08[c]
RCG	35.4	50.4	3.1	5.2	31	51	87	100	0.13	0.11

[a] Based on total initial sugars available for the fermentation.
[b] Based on consumed sugars.
[c] Value after 48 h of reaction.

The highest ethanol yield is from alfalfa and barley straw but the lowest are from timothy and RCG (table 4). Belkacemi et al. (1998) concluded that based on their results for barley straw (yield, pre-treatment and other method used), a yield of 190 litres ethanol/t dry biomass could be achieved from hydrolysed forages and agricultural residues.

Alvo and Belkacemi (1997) examined the enzymatic saccharification of milled timothy and alfalfa. Since size reduction is a crucial step in the conversion of lignocellulosic biomass to fuel they wanted to compare preliminary shredding and milling to more extensive mechanical reduction, in order to evaluate the energy efficiency. For this project, they used timothy at two stages of maturity and alfalfa at one stage of maturity. The HLs from the late-season timothy was then fermented with *Saccharomyces cerevisiae.* Their results showed that the timothy HLs was fermented to 88% of theoretical yield with the addition of nutrients and 80% without the addition of nutrients. They discussed that their data showed that both the biomass type and maturity has great effect on the outcome of the HLs and it is easier to hydrolyse leaf material compared to stem material.

1.9.6 Arable Land in Iceland

There has been discussion on how much biofuel is possible to produce in Iceland (H. Bjornsson, 2007; Sveinsson & Hermannsson, 2010). Iceland is 103.000 km^2 (Landmælingar Íslands, 2014) but only a small portion of that land area is possible to cultivate. There have been made few attempts to categorize land in Iceland and find out how much possible arable land there is.

In 1961 the National Land Survey of Iceland (NLSI) published estimates on total vegetation cover in Iceland. This estimation was based on maps at the 1:100,000 scale, however some of the maps used for this estimation were up to 60 years old. Based on this information, vegetated land below 200 MASL was about 13,718 km² and 9,112 km² was arable deserts. This estimation was then used in report made in 1976 by a research committee for the Icelandic government, about the development of agriculture. It was estimated that of the arable deserts, it would be possible that about 5,000 km^2 of deserts would be possible to convert to arable land that could give acceptable yield. Given that approximately 20% of the potential arable land will be needed and used for construction, roads, buildings and etc. the final estimation leaves potential arable land to about 15,000 km^2 (Snaebjornsson et al., 2010).

Traustason and Gisladottir (2009) used Geographic Information Systems (GIS) to estimate potential arable land. Their estimation is based on the Icelandic Farmland Database and the European land cover project, Coordination of Information on the Environment (CORINE). The Icelandic Farmland Database (Icelandic: Nytjaland) is a project that started in 1999 to collect information on land and land use for an online database. This project is cooperation between the Agricultural University of Iceland, the Ministry of Fisheries and Agriculture, the Farmers Association of Iceland and Soil Conservation Service of Iceland. The project is based on satellite imagery from Landsat 7 and SPOT 4 and 5 (Nytjaland, n.d.). The purpose of the CORINE project is to collect information about land use and observe changes over period of time. The CORINE project is carried out at the same time in most countries of Europe (Landmælingar Íslands, 2010). Traustason and Gisladottir (2009) used the following assumptions:

- The area must be in the categories: grassland, richly or poorly vegetated land or semi-wetland.
- Slopes are less than 10° and elevation below 200 MASL.
- Land outside protected areas near roads and urban areas, but not further away than 2 km from main roads.
- Protected areas are excluded.

They concluded that given these assumptions, potential arable land is approximately 6,150 km^2, or 6.0% of Iceland. However, they notify that their estimation did not take into

account climate or soil type and therefore there could be some areas that are not suitable for cultivation.

Áslaug Helgadóttir and Jónatan Hermannsson estimated potential arable land as a part of a report done for the Icelandic Ministry of Fisheries and Agriculture (Snaebjornsson et al., 2010). They focused on the size of good quality arable land and used following assumptions:

- Land must be below 200 MASL, exceptions made where there are fields in use above 200 MASL.
- Soil must be deeper than 30 cm to make ploughing possible.
- If the land type is wetland, it must be possible to drain without major difficulties.
- Sands and river sandbanks are included but not dunes or sands by glacial rivers.
- Minimum size of each field should not be less than 3 hectares.
- Ditches for drainage can be in the 3 hectares fields but the fields must be in continuity.

This estimation resulted in about 6,000 km^2 of good arable land available (Snaebjornsson et al., 2010).

Sveinsson and Hermannsson (2010) estimated the potential arable land with assistance from local agricultural advisors around the country and also using estimation previously done by the Icelandic Biomass Company (H. Bjornsson, 2007). Sveinsson and Hermannsson (2010) were specially focusing on arable land fit for large-scale production of biomass. Since there was no information available on how much area would be suitable for that purpose, they contacted agricultural advisors from each part of the country to get estimation from people well acquainted with each area. The objective was to find out the total size of area suited for a large-scale production that is still not cultivated. It had to be in continuity and easy to cultivate without too much costs. These factors are important for good productivity (Sveinsson & Hermannsson, 2010). The criteria were that each cultivation unit should be at least 10 hectares in size, without too many rocks, and soils with minimum fertility and depth (Þóroddur Sveinsson, personal communication, January 6th, 2015). These assumptions eliminate large areas of the lowland. Sveinsson and

Hermannsson (2010) concluded that potential arable land in Iceland, not in use and suitable for large-scale biomass production was approximately 420 km^2.

The exact area of potential arable land is unknown but there is data available for the area that has been cultivated. According to report done for the Icelandic Ministry of Fisheries and Agriculture, Áslaug Helgadóttir and Jónatan Hermannsson estimated that cultivated land in Iceland was approximately 1.200 km^2, type of cultivated land is listed in table 5 (Snaebjornsson et al., 2010).

Table 5. Estimation of cultivated land in Iceland (Snaebjornsson et al., 2010).

Use of cultivated land	Area (km^2)
Old hayfields	700
Hayfields, 1-6 years old	360
Other forage crops	80
Grains (barley mostly)	50
Horticulture	10
Total	**1200**

1.10 Bioenergy Residues

There are many ways to convert biomass into energy. It can be achieved by the use of various technologies, from different types of biomass. Bioenergy production generates residues or by-products (Cayuela et al., 2010). These by-products are for example digestates from biogas, seed meals from biodiesel, distiller's grain solids from ethanol, and bio-char from pyrolysis. It is possible that many of these by-products, as they are produced on an increasingly larger scale, will be used as soil amendments or fertiliser on agricultural land (Gell et al., 2011). With an expansion in a bioenergy production questions have been raised concerning the effects it has on land use and the environment (Cayuela et al., 2010).

The chemical and physiological properties of bioenergy by-products are very different depending on the type of biomass and method used for the production. Generally these residues still contain large amounts of C and many other mineral nutrients. These nutrients could be recycled by using these products as soil amendments or fertilisers (Cayuela et al., 2010).

Several studies have analysed greenhouse gas (GHG) balance of bioenergy production, however, there has been little focus on the environmental impact of bioenergy by-products. Soil carbon (C) and nitrogen (N) cycles are not easily understood (Cayuela et al., 2010), but bioenergy will probably have some effect on that matter. Farrell et al. (2006) highlighted the importance to take into account the by-products effect on net energy and GHG calculations (Farrell et al., 2006).

Residues from Ethanol Production from Lignocellulosic Biomass

There are few studies and little data on the use or environmental effect from bioethanol residues. Additionally, the use of residues from ethanol production from lignocellulosic biomass as fertiliser or for soil amendments has not been investigated.

The main solid residues from the ethanol production process from lignocellulosic biomass beside ash (with various mineral nutrients) are lignin and non-soluble cell wall carbohydrates and organically bound N. The amount and quality of the residue varies between feedstock types and also between methods used for the process. It is possible to use the lignin for high-octane hydrocarbon fuel additives or to replace phenol in phenol formaldehyde resins. It is also possible to use the residue as burning material for heat and/or electricity production (Hamelinck et al., 2005).

2. Aims and Benefits of this Study

The aim of this study is to determine ethanol yields and other end products after fermentation with selected organisms using timothy grass cut at four different maturity stages as substrates. The results will be used to calculate how much ethanol can possibly be produced in Iceland, by methods used in this research. Possible use of the residue that is produced in the process will also be discussed.

1. Research Question

What effect has the harvest time/growth stage of timothy on ethanol yield?

2. Research Question

What organisms have the best production efficiency (L ethanol/t biomass) and highest ethanol yield/area (L ethanol/ha)?

3. Research Question

How much ethanol could be produced from timothy in Iceland, using these microorganisms?

Benefits of this Research

Results will be used to determine how much ethanol can be produced from timothy in Iceland. The results should be useful to determine the best harvest time of timothy for ethanol production and what organisms to use to get the best efficiency. Additionally, it would be interesting to see if it is possible to find some use for the residue produced in the process of the biomass.

It is possible that biomass production can be an addition to Icelandic agriculture. Biomass production from timothy is especially interesting because it is the most common forage crop in Iceland.

3. Materials and Methods

The timothy used for this research was harvested in a grass field at Möðruvellir Experimental Station in Hörgárdalur (65°46.239', 18°15.080' (65,7707, 18,2513)) in 2014. Möðruvellir is a satellite facility from the Agricultural University of Iceland. All batch experiments were conducted in laboratories at the University of Akureyri in October and November 2014.

3.1 Medium

Two types of basal medium (BM) were used for the experiments, BM for anaerobic cultures and BM including peptone for the aerobic cultures. Here after, it will be referred as BM whether it is for anaerobic or aerobic cultures. The content of the BM is shown in table 6.

Table 6. The contents of the BM.

Compound	Amount in 1 liter of medium
Distilled H_2O	885 mL
1 M Phosphate buffer (pH 7)	50 mL
Yeast extract (YE)	2 g
Peptone *	2 g
Resazurine (5 ppm)	5 mL
C_1 Solution	50 mL
NaCl	0.3 g
NH_4Cl	0.3 g
$CaCl_2+2H_2O$	0.11 g
$MgCl_2+2H_2O$	0.1 g
$FeCl_3+4H_2O$	2 mg
EDTA	0.5 mg
$CuCl_2$	0.03 mg
H_3BO_3	0.05 mg
$ZnCl_2$	0.05 mg
$MnCl_2+4H_2O$	0.05 mg
$(NH_4)_6MO_7O_{24}+4H_2O$	0.05 mg
$AlCl_3$	0.05 mg
$CoCl_2+6H_2O$	0.05 mg
Vitamin solution	1 mL
Biotin	2.0 µg
Folic acid	2.0 µg
Pyridoxine-HCl	10.0 µg
Thiamine-HCl x 2 H_2O	5.0 µg
Riboflavin	5.0 µg
Nicotinic acid	5.0 µg
D-Ca-pantothenate	5.0 µg
Vitamin B12	0.1 µg
p-Aminobenzoic acid	5.0 µg
Lipoic acid	5.0 µg
C_2 Solution	10 mL
$NaHCO_3$	0.72 g
$C_3H_7NO_2S+HCl+H_2O$	5 mg
Na_2S+9H_2O	0.24 g
* Only in BM for aerobic cultures	

The medium was prepared by mixing distilled water, YE, the phosphate buffer and resazurin solution in an Erlenmeyer flask. The medium was boiled for 5 minutes (colour of the medium went from blue to pink), poured into a screw cap flask and cooled down rapidly in ice bath. The medium for anaerobic cultures was sparged with N_2 (< 5 ppm O_2) while cooled down. Serum bottles (24.4 mL) were flushed with N_2 for 60 seconds and then the medium was dispensed into the bottles (L:G ratio 1:1) and sparged with N_2 for 20 seconds. Then the bottles were closed with butyl rubber septa and sealed with aluminium crimp caps. The medium for aerobic cultures was dispensed into serum bottles (24.4 mL, L:G ratio 1:1) and closed with cotton plug and aluminium foil. Finally, media was autoclaved at 121°C for 60 minutes. The remaining components of the medium, C_1 (for aerobic and anaerobic cultures) and C_2 (only for anaerobic cultures), were added into each bottle after autoclaving just prior to inoculation. All solutions added to the medium after autoclaving were filter sterilized through a sterile 0.45 μm syringe filter (Whatman, PES).

3.1.1 Organisms and Fermentation

Inoculation volume was 2% (v/v) for all organisms and was taken from exponential growth phase of stock culture. Six different organisms were used for each set of experiments. Anaerobic bacteria used were *Clostridium thermocellum* (DSM 1237, 55°C), *Thermoanaerobacter ethanolicus* (DSM 2246, 65°C) and *Thermoanaerobacter* strain J1 (65°C) (Jessen & Orlygsson, 2012). Facultative aerobic organisms used were *Kluyveromyces marxianus* (ATCC 10022, 30°C), *Saccharomyces cerevisiae* (DSM 1334, 30°C) and *Zymomonas mobilis* (DSM 424, 30°C). All reference strains where obtained from either the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) or from the American Type Culture Collection (ATCC). All organisms were cultivated at pH 7. Strains were cultivated for 5 days at which time 1 mL of liquid sample and 0.2 mL of gas was removed and analysed. Additional sample was taken after one day for aerobically grown microorganisms.

3.1.2 Ethanol Production from Simple Substrates

This experiment was set out to gain knowledge of each organism's end product formation patterns on major substrates found in lignocellulosic biomass. All microorganisms were cultivated on six different sugars (glucose, xylose, mannitol, arabinose, L-rhamnose and D-cellobiose) at concentration of 20 mM/L as well as on avicel (cellulose) and starch (2%

w/v) in batch culture. Strains were also cultivated on 2 g/L of YE as a control. All experiments were performed in duplicate.

3.2 Ethanol Production from Timothy

3.2.1 Timothy Samples

Timothy samples were collected from a 3-year-old timothy field (variety; Vega (Graminor)) at Möðruvellir and harvested at four different primary (1st cut) harvest times (maturity stages) in 3 replicates (plots) each time. The experimental plots received in early May approximately 100 kg N/ha, 15 kg P/ha and 41 kg K/ha in a combined commercial fertiliser. All experimental plots were 2x6 m, gross size. Harvested plots were 1.2 m wide but the length did vary from 2.3 m to 3.5 m between plots. The timothy leave area cover in the plots was >90%. Other noticeable grass species were meadow foxtail (*Alopecurus pratensis* L.) and Meadow fescue (*Festuca pratensis* Huds.) and few dandelions (*Taraxacum officinale*). These species were set aside before sampling. The samples for analysis were weighted and dried in an oven at 50-60°C for three days before put in dry storage at room temperature.

3.2.2 Preparation of Hydrolysates

Timothy samples were ground with Aarslev Maskinfabrik hammer mill with 1 mm sieve. Whatman paper was cut down in Waring blender into small pieces. The triplicate samples for each harvest time were combined into one sample. Four timothy samples (DM 0.93) and Whatman paper were weighted into a screw cap flask in two different stock concentrations, 25 g/L and 50 g/L for each sample. Approximately 230 mL of 0.5% (v/v) sulphuric acid (H_2SO_4) was added to the samples and autoclaved at 121°C for 60 minutes and put in a fridge overnight at 5°C. HLs pH was adjusted to pH 5. Enzymes were then added (1 mL/1L HLs), ß-glucosidase (cellobiase) from *Aspergillus niger* (Sigma, Product 49291, enzyme units >750 U/g) and cellulase from *Trichoderma reesei* (Sigma, Product C2730, enzyme units >700 U/g) and incubated on an orbital shaker (45-50°C, 150 rpm) for 72 hours. Next, HLs were centrifuged at 4700 rpm for 5 minutes. HLs were then pH adjusted to pH 7 and then vacuum filtered sequentially through 11 µm (Whatman #1 filter paper), 5 µm (nylon), and 0.45 µm (polyamide) filters; the volume was adjusted to 250 mL

with dH_2O prior to being filter sterilized into N_2-flushed serum bottles through a sterile 0.45 μm syringe filter (Whatman, PES).

3.2.3 Fermentation of Hydrolysates

The HLs were added to the serum bottles in three different final concentrations (timothy with DM 0.93), 2.5 g/L, 5.0 g/L (from 25 g/L stock solution), and 25 g/L (from 50 g/L stock solution) just prior to inoculation. Inoculation was done as previously described. All experiments were performed in duplicate.

3.2.4 Timothy Samples without Acid/Enzyme Pre-treatment

The four types of timothy samples plus the Whatman paper, in three different concentrations; 2.5 g/L, 5 g/L and 25 g/L were prepared for extra set of experiment with the use of *Clostridium thermocellum*. Both the timothy and Whatman paper samples were weighted directly into the serum bottles without any acid/enzyme pre-treatment. Everything else was done as described earlier.

3.3 Analytical Methods

3.3.1 Analysis of End Products and pH

Hydrogen was measured by a Perkin Elmer gas chromatograph as previously described by Orlygsson and Baldursson (2007). Ethanol and volatile fatty acids were analysed by the use of Perkin Elmer Clarus 580 gas chromatograph using an FID detector with 30 metres DB-FFAP capillary column (Agilent Industries Inc, Palo Alto, CA, US). The final pH of the cultures was measured by using a Thermo Scientific 4 star pH meter.

3.3.2 Analysis of Sugars

The amounts of total sugars, cellobiose, glucose and xylose in HLs (stock solutions), prior to fermentation, was measured with high performance liquid chromatography (HPLC) with a refractive index detector (40°C). The column used for the HPLC runs was a Bio-Rad Aminex HPX-87H (300 mm × 7,8 mm) at ambient temperature. A 5 mM H_2SO_4 solution was used as eluent, with a flow rate of 0.6 mL/min; injection volume was 20 μL. Each sample was diluted up to 5 times prior to injection.

Total sugars in timothy HLs samples after five-day fermentation, were determined by using Anthrone Method as described by Aminoff et al. (1970) adapted for use in microtiter

plates. Colorimetric assay was used to determine xylose concentration based upon the method of Eberts et al. (1979) adapted for use in microtiter plates.

3.3.3 Analysis of the Timothy and Hydrolysate Residue

The timothy samples and the HLs residues were sent to the Agricultural University of Iceland, for analysis.

The HLs residue after the first filtration was mostly dried at 50-55°C but then at 60°C in hot-air-oven until steady weight was reached, after about 32 hours. The residue samples were not grinded further but crushed by hands through 1 mm sieve.

C and N analysis was performed with Dumas method in the equipment Elementar varioMax CN (Elementar Anlysensysteme GmbH, Hanau Germany). Cellulose and aNDF analysis was done with the Ankom method with the instrument Ankom 200 (Ankom Technology, Macedon NY, USA). The method used is based on the primary process with the addition of amylase and sulphite, which has become a standard addition and commonly used in all analytical laboratories.

DM was measured in grinded samples at the same time when weighted for previously mentioned measurements. The values were adjusted for 102°C drying in hot-air-oven over night. Ash was obtained by burning the samples at 550°C and then left in oven for 4 hours after that temperature was reached.

3.4 Statistical Analysis and Calculations

Statistical analysis was carried out in JMP® (© 2012 SAS Institute Inc.: www.jmp.com). Analysis of variance (ANOVA) models with blocked replicates were used to calculate means for treatments, interactions between treatments (if applicable), mean standard errors and probability ratios. Statistical significances between treatment means were determined with the aid of Student's t tests (ST). Other calculations were performed in Microsoft Excel for Mac (2011).

4. Results

4.1 Ethanol Production from Simple Substrates

The first experiments were conducted to investigate the strains ethanol production from simple substrates. All six organisms were cultivated with various carbon sources (sugars (20 mM), starch and avicel (2% w/v), and the end products analysed.

The results for *C. thermocellum* are shown in figure 13. No production above controls (YE only) was observed on any of the carbon sources, except on avicel, which resulted mainly in ethanol production (23.8 mM) but also acetate (9.0 mM) and hydrogen (4.7 mmol/L).

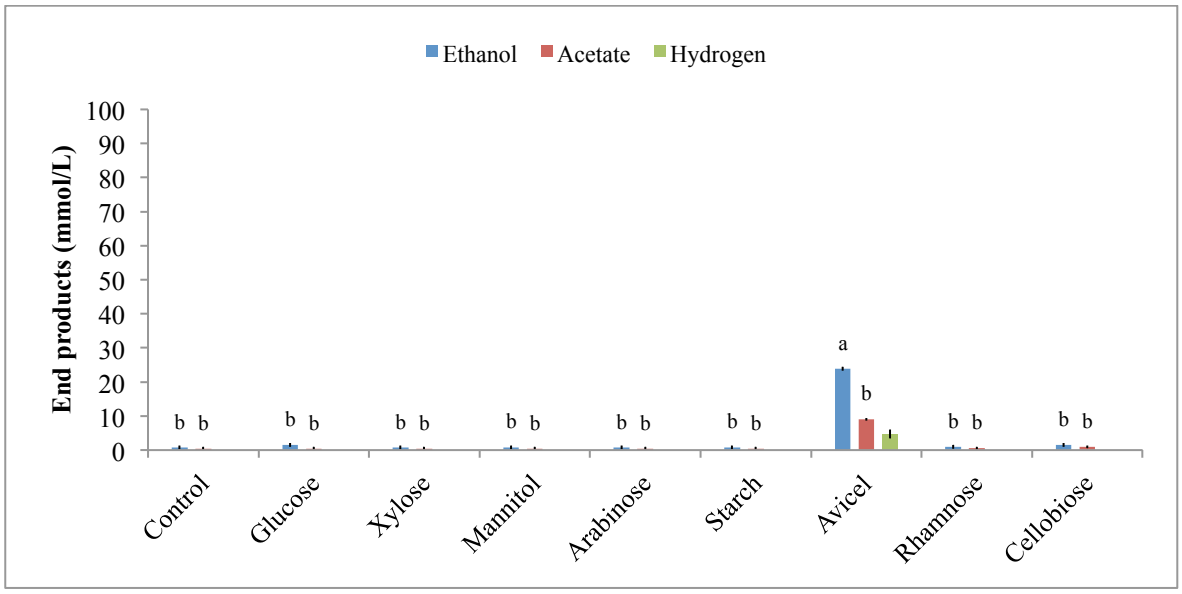

Figure 13. End products formation on simple substrates by *Clostridium thermocellum*. Means with different letters (abc) within the same end product are significantly different according to Student's t test (ST) at 95% confidence level. Bars correspond to standard error (SE) of mean.

T. ethanolicus produced ethanol, acetate and hydrogen from glucose, xylose, mannitol, and starch (Figure 14). Substrates revealing the highest ethanol production were starch and mannitol, 30.8 mM and 27.9 mM, respectively. From substrates degraded, acetate was always found to be in lower concentrations as compared to ethanol, but proportionally highest on xylose (ratio ethanol/acetate = 1.08). Hydrogen was the highest end product observed for xylose 17.1 mmol/L but the highest concentration of hydrogen was from mannitol (21.3 mmol/L), however, not significantly different from the hydrogen production from starch (18.8 mmol/L).

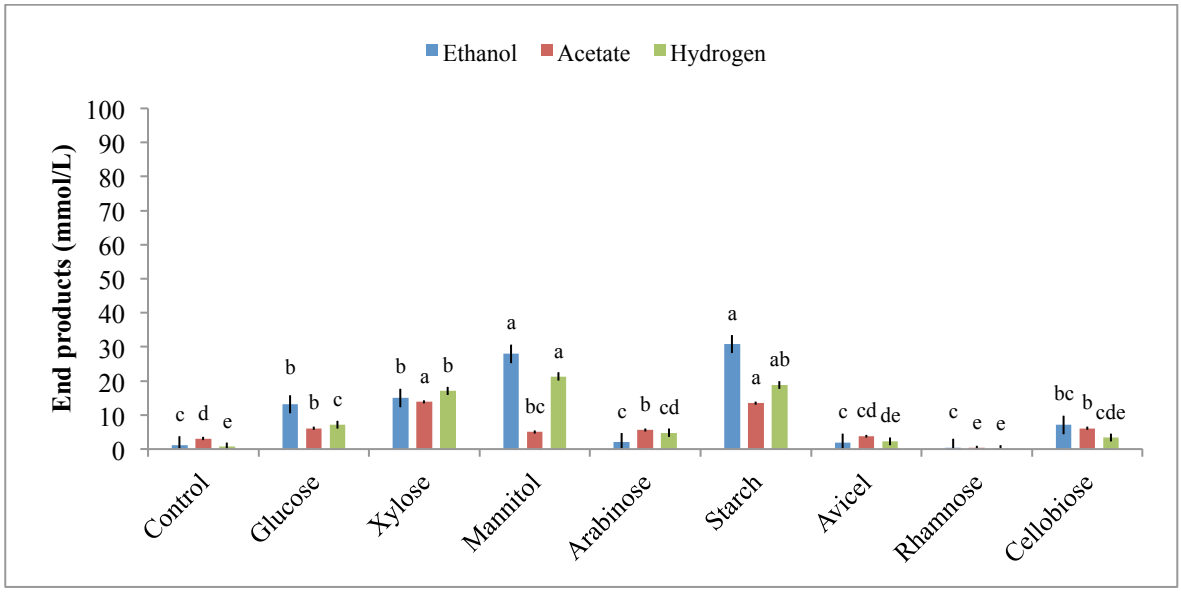

Figure 14. End products formation on simple substrates by *Thermoanaerobacter ethanolicus*. Means with different letters (abc) within the same end product are significantly different according to ST at 95% confidence level. Bars correspond to SE of mean.

Figure 15 displays the results for *Thermoanaerobacter* strain J1. This strain was able to produce mainly ethanol but also small amounts of acetate and hydrogen from glucose, xylose, mannitol, starch, and cellobiose. The highest concentration of ethanol was observed on starch, 96.7 mM. Among the sugars, the highest concentration of ethanol was observed on glucose, 36.7 mM. Highest amounts of acetate were produced from xylose, 13.9 mM and most hydrogen was produced on mannitol, 21.3 mmol/L.

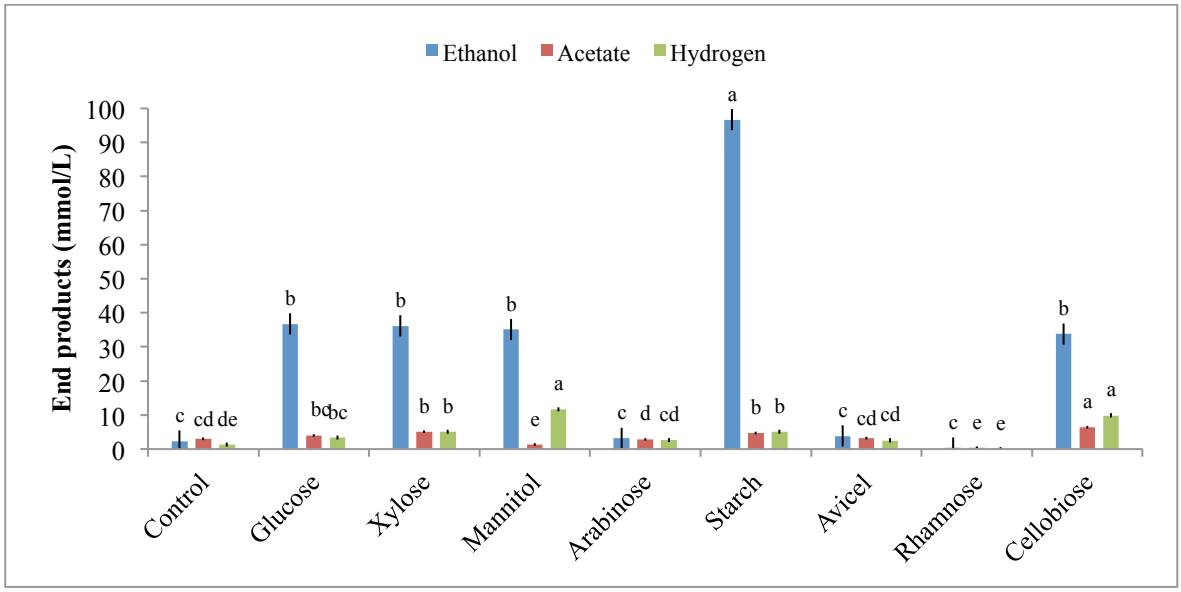

Figure 15. End products formation on simple substrates by *Thermoanaerobacter* strain J1. Means with different letters (abc) within the same end product are significantly different according to ST at 95% confidence level. Bars correspond to SE of mean.

Figure 16 shows the end product formation from simple substrates by *Z. mobilis*, after both one day and five days. The one-day samples showed that the strain produced ethanol from glucose (12.7 mM), mannitol (2.5 mM) and cellobiose (7.7 mM). However the five-day samples resulted in no ethanol production above controls, only from glucose (11.3 mM). Surprisingly, the main end product for the five-day sample on glucose was acetate, 16.5 mM. The ethanol concentration from cellobiose was higher for one-day samples (7.7 mM) compared to five-day samples (0.4 mM). On the other hand, the acetate concentration was much higher for the five-day samples compared to one-day, 8.9 mM and 1.4 mM, respectively. Ethanol production was very little from other substrates; xylose, arabinose, starch, avicel, and rhamnose, for one-day samples, and not above production from control.

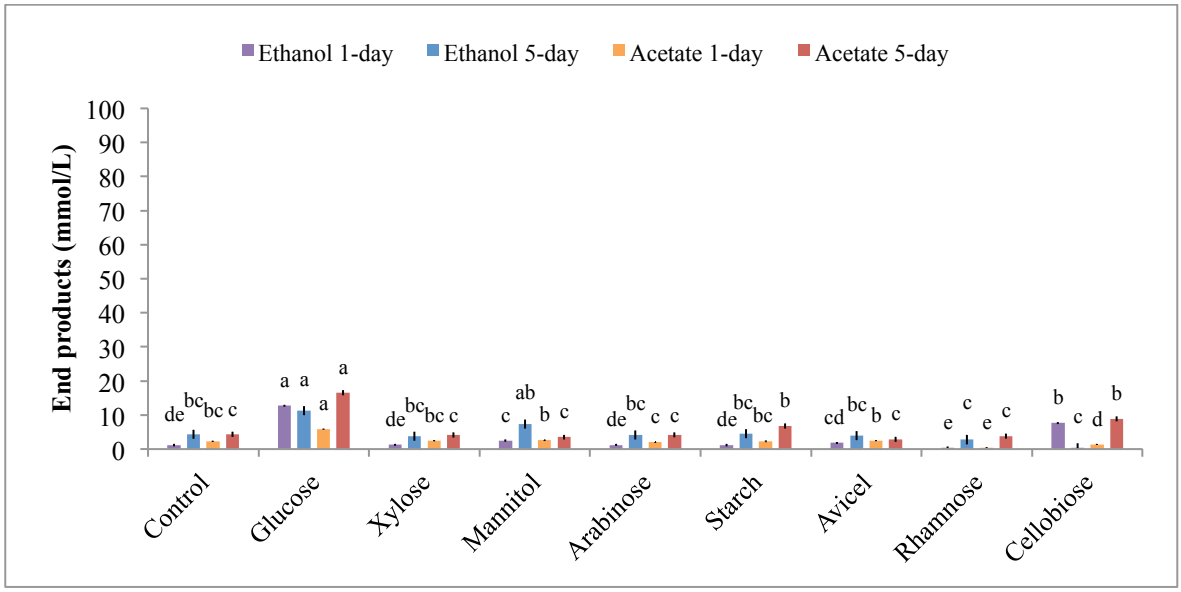

Figure 16. End products formation on simple substrates by *Zymomonas mobilis* after one and five days. Means with different letters (abc) within the same end product and day are significantly different according to ST at 95% confidence level. Bars correspond to SE of mean.

Results for *K. marxianus* ethanol production from simple substrates are shown in figure 17. The only ethanol and acetate production observed was on glucose and very little on cellobiose. The ethanol concentration from glucose was higher for the one-day sample compared to the five-day sample, 35.3 mM and 13.0 mM respectively. The acetate concentration was however higher for the five-day sample (9.3 mM) compared to the one-day sample (2.6 mM).

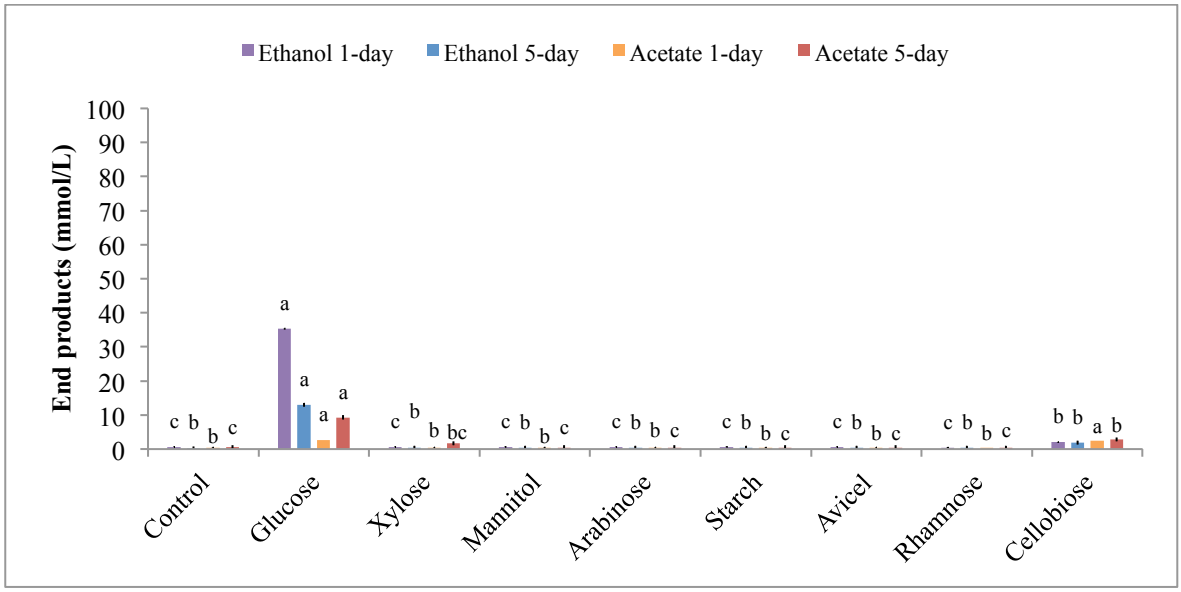

Figure 17. End products formation on simple substrates by *Kluyveromyces marxianus* after one and five days. Means with different letters (abc) within the same end product and day are significantly different according to ST at 95% confidence level. Bars correspond to SE of mean.

Figure 18 displays the results for *S. cerevisiae,* ethanol production from simple substrates. The strain produced ethanol from glucose and cellobiose. The ethanol concentration on both glucose and cellobiose was higher for five-day sample compared to samples one-day samples. The highest ethanol concentration was from five-day sample cellobiose (37.6 mM). The ethanol concentration for one day on glucose was 37.2 mM and 36.9 mM for the five-day sample. There was also acetate production above controls from both one-day (6.9 mM) and five-day (12.4 mM) samples on cellobiose and also from five-day samples on starch (10.9 mM) and avicel (7.3 mM).

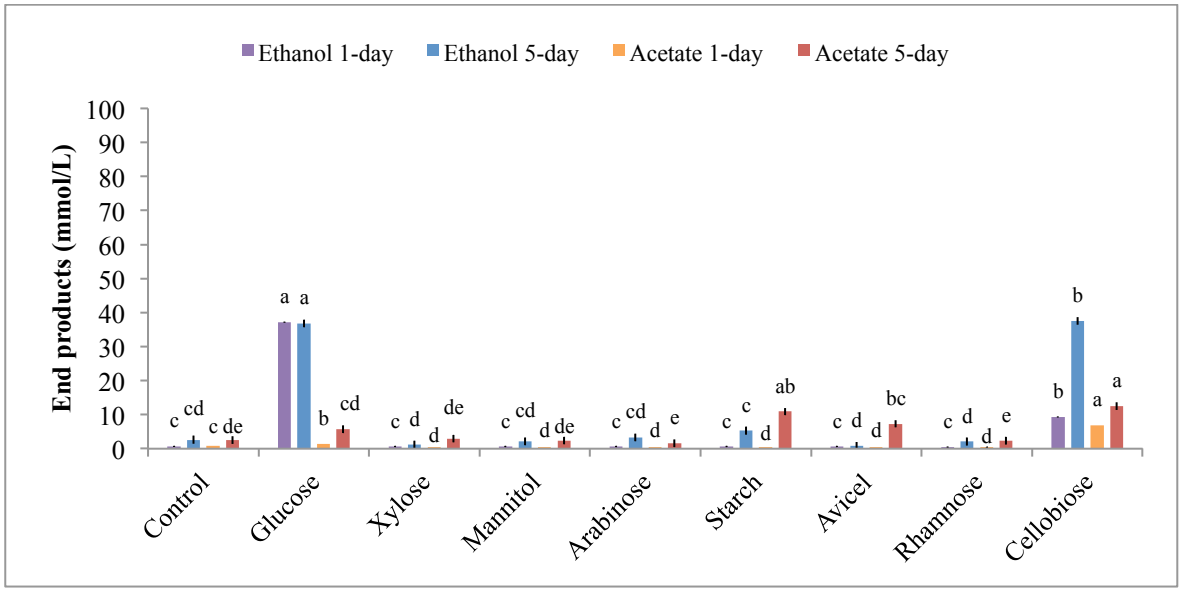

Figure 18. End products formation on simple substrates by *Saccharomyces cerevisiae* after one and five days. Means with different letters (abc) within the same end product and day are significantly different according to ST at 95% confidence level. Bars correspond to SE of mean.

4.2 Ethanol Production from Timothy

The main objective of this project was to investigate the ethanol production of all selected strains from timothy HLs. All six strains were cultured with three different HLs concentrations, comparing four different harvest dates of the timothy. Additionally, WP was used for comparison.

4.2.1 Timothy Samples

The weather data for Möðruvellir Experimental Station for the summer 2014 is shown in table 7. Total GDD were 1,290 from beginning of May till the end of August.

Table 7. Meteorological data in 2014 from the timothy field at Möðruvellir (65°46.239', 18°15.080').

	Temperature (°C)			GDD	Precipitation
Month	Air (2 m)	Surface	Root zone	(Air)	(mm)
May	7.0	8.9	7.4	217	10
June	12.0	15.2	13.8	361	26
July	12.5	14.7	14.4	387	61
August	10.5	12.0	12.3	325	16

The first harvest time was June 4th with total GDD of 265 until harvest. The second harvest time was June 16th with total GDD of 410, the third harvest time was in July 10th with 703 GDD and then the forth August 11th with 1,081 GDD.

Timothy Yield

The effect of growth stage (harvest dates) on timothy DM primary yield is shown in figure 19. The DM yield increased with maturity till full heading but stagnated during blooming. The timothy yield for the first harvest time was 2.1 t DM/ha, 3.8 t DM/ha for the second, 7.0 t DM/ha for the third and 7.1 t DM/ha for the fourth harvest.

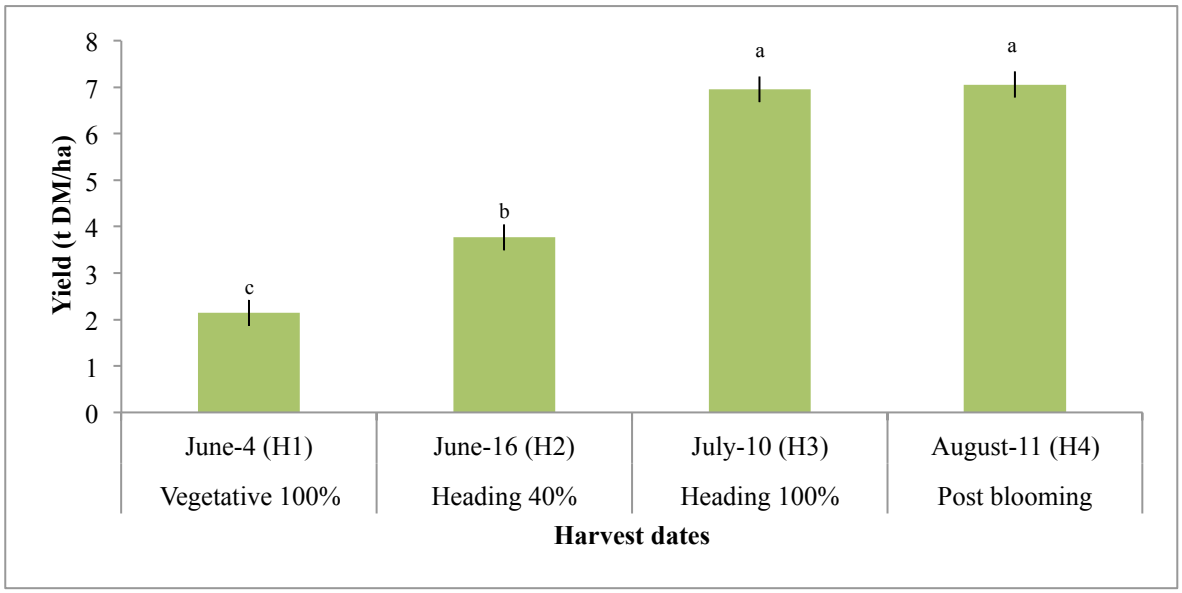

Figure 19. Effect of four different harvest dates on timothy primary yield. Means with different letters (abc) are significantly different according to ST at 95% confidence level. Bars correspond to SE of mean.

Timothy Hay Samples

The results for timothy samples analysis are shown in table 8. The samples were analysed for total DM, organic matter, ash, NDF, N, C, and ruminal dry matter digestibility (DMD). There was a significant difference for the content of N, and the C/N ratio, between all harvest times. The N content declined rapidly with increased maturity, from 2.8% of DM (H1) to 0.7% of DM. For C, H4 was the only harvest time significantly different, resulting in 45.5% of DM, H1, H2, and H3 ranged between 46.3% and 46.6% of DM. Therefore, the C/N ratio increased with maturity, from 17:1 (H1) to 63:1 (H4). Organic matter resulted in

higher proportion and the ash in lower, for the latter harvest days. NDF was significantly lowest for H1 (46.3%).

Table 8. Timothy hay samples analysis. Values are shown in % of DM. Means within the same column with different letters (abc) are significantly different according to ST at 95% confidence level.

Harvest time	Organic matter		Ash		NDF		N		C		C/N	
	Mean	ST	Mean	ST	Mean	ST	Mean	ST	Mean	ST	Mean	ST
1	93.6	c	6.4	a	46.3	b	2.8	a	46.6	a	17.0	d
2	94.2	b	5.8	b	56.7	a	1.9	b	46.6	a	24.4	c
3	94.8	a	5.2	c	59.5	a	1.2	c	46.3	a	39.0	b
4	94.6	a	5.4	c	55.2	a	0.7	d	45.5	b	62.7	a
Mean	94.3		5.7		54.4		1.6		46.3		35.8	
SE of mean	0.13		0.13		1.53		0.09		0.15		1.50	

The DMD of the timothy samples is shown in figure 20. The DMD declined with increased maturity, H1 (74% DMD), H2 (69.7% DMD), H3 (61.7% DMD) and H4 (56% DMD). The measurement of DMD for the last harvest date (H4) was outside conventional measurement.

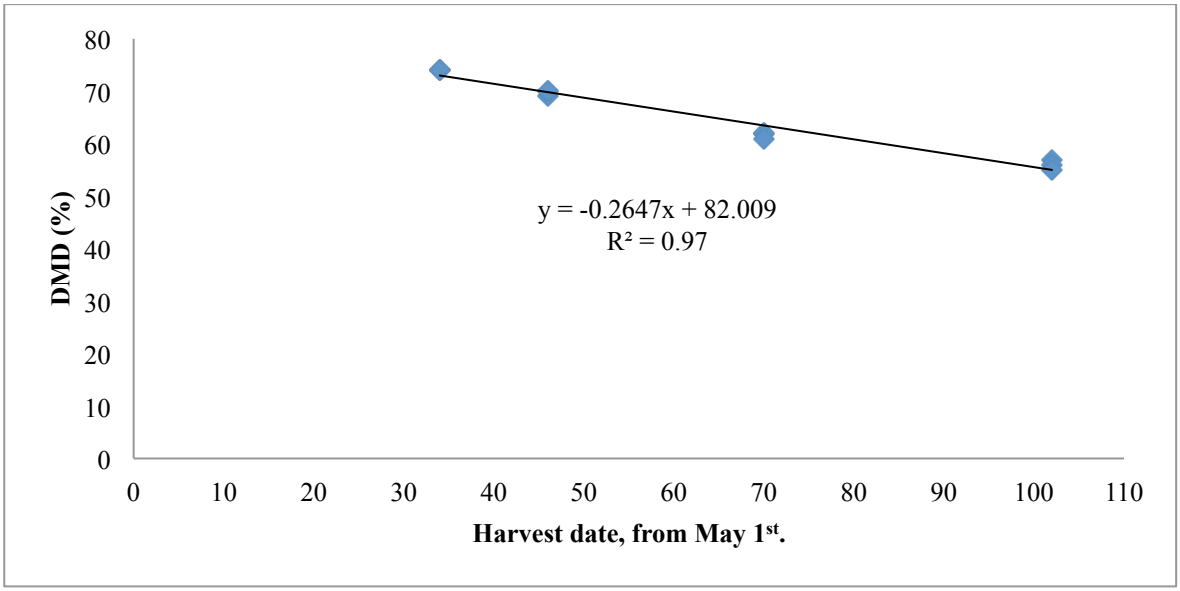

Figure 20. Harvest time effect on DMD decline of timothy primary cut.

4.2.2 Initial Sugar Concentrations

The total sugar concentrations from the timothy HLs ranged from 2.6 mM/g to 3.1 mM/g (table 9). The only significant difference found between harvest times, for total sugar concentration, was between HLs of H2 and H3. The glucose concentration ranged from 1.1 to 2.9 mM/g, highest for H4, however only significantly different from H2. For xylose concentration, H3 and H4 were significantly different. As was mentioned in the literature review, the theoretical yields of ethanol is 2.0 mol from 1 mol hexose and 1.66 mol for pentose. Theoretical ethanol yield from the HLs is shown in table 9. The highest theoretical ethanol yield from the timothy HLs was from H4, 5.5 mM/g.

Table 9. Initial sugar concentrations of HLs. Means (main effects across HLs concentrations) within the same column with different letters (abc) within substrate are significantly different according to ST at 95% confidence level.

	Initial concentration (mM/g)								Theoretical ethanol yield from HLs (mM/g)		
HLs type	Total sugar	ST	Cellobiose	ST	Glucose	ST	Xylose	ST	Glucose	Xylose	Glucose + xylose
WP	3.0	ab	0.03	ab	2.9	ab	0.0	c	5.9	0.0	5.9
H1	3.0	ab	0.01	bc	1.4	bc	1.6	b	2.8	2.7	5.4
H2	3.0	ab	0.04	a	1.5	a	1.4	b	3.0	2.3	5.4
H3	2.6	b	0.00	c	1.1	c	1.5	b	2.2	2.4	4.7
H4	3.1	a	0.00	bc	1.1	bc	2.0	a	2.1	3.3	5.5
Mean	2.9		0.02		1.6		1.3				
SE of mean	0.1		0.01		0.1		0.1				

4.2.3 Sugar Degradation

Table 10 shows how effective different strains were on sugar degradation. The proportion of sugar residues was not affected by harvest time or HLs concentration with the exception of *T. ethanolicus*. *T. ethanolicus* sugar degradation was affected by HLs concentration ($p=0.0049$). HLs with 25 g/L concentration had lower sugar degradation compared to 2.5 and 5.0 g/L or 68% vs. 88% and 89%, respectively. The sugar degradation (-7%) of *C. thermocellum* was significantly different from degradation by other strains and suggests that the total sugar availability increased during the fermentation. The total sugar degradation (62%) of *Z. mobilis* was significantly different from the total sugar degradation of other strains. The results showed that the strains did not degrade all sugars

in HLs, but the best sugar degradation was by *Thermoanaerobacter* strain J1 87% of initial sugar concentration, then *S. cerevisiae* and *K. marxianus,* 80% and 79%, respectively.

Table 10. The main effect of strain on sugar residues and proportional degradation (main effects across HLs concentrations). Means within the same column with different letters (abc) within substrate are significantly different according to ST at 95% confidence level.

Strain	Glucose (mM/g)	ST	Xylose (mM/g)	ST	Total sugar (mM/g)	ST	Sugar degradation	ST
C. thermocellum	1.8	a	1.3	a	3.1	a	-7%	c
T. ethanolicus	0.3	c	0.5	b	0.8	bc	72%	a
Thermoanaerobacter strain J1	0.2	c	0.2	c	0.4	c	87%	a
Z. mobilis	0.8	b	0.3	bc	1.1	b	62%	b
K. marxianus	0.1	c	0.5	b	0.6	bc	79%	a
S. cerevisiae	0.2	c	0.4	bc	0.6	bc	80%	a
Mean	0.6		0.5		1.1		62%	
SE of mean	0.19		0.11		0.21		6%	

4.2.4 Ethanol Production from Whatman Paper

The results on mean ethanol production (across three HLs concentrations, 2.5, 5.0 and 25 g/L) from WP HLs are shown in table 11. One-day samples for yeasts, *K. marxianus* and *S. cerevisiae* resulted in highest ethanol concentration from WP, 3.9 mM/g and 3.7 mM, respectively. *Thermoanaerobacter* strain J1 (3.1 mM) produced more ethanol than *T. ethanolicus* (1.8 mM).

Table 11. Mean ethanol production from WP (main effects across HLs concentrations). Means with different letters (abc) are significantly different according to ST at 95% confidence level.

Organism	Mean ethanol production (mM/g)	ST
Clostridium thermocellum	0.4	d
Clostridium thermocellum no pre-treatment	0.1	d
Thermoanaerobacter ethanolicus	1.8	c
Thermoanaerobacter strain J1	3.1	b
Zymomonas mobilis 1-day	0.5	d
Zymomonas mobilis 5-day	0.6	d
Kluyveromyces marxianus 1-day	3.9	a
Kluyveromyces marxianus 5-day	2.2	c
Saccharomyces cerevisiae 1-day	3.7	ab
Saccharomyces cerevisiae 5-day	3.1	b
Mean	1.8	
SE of mean	0.23	

4.2.5 Ethanol Production from Timothy Hydrolysates

The results for *C. thermocellum* ethanol production from the different HLs concentrations are shown in figure 21. The highest ethanol concentration was 18.8 mM, obtained from the 25 g/L concentration of H2. Highest ethanol yields were observed on the 5 g/L concentration of H2 or 1.2 mM/g DM (control values (growth on YE) subtracted). Acetate was also formed, but in low concentration.

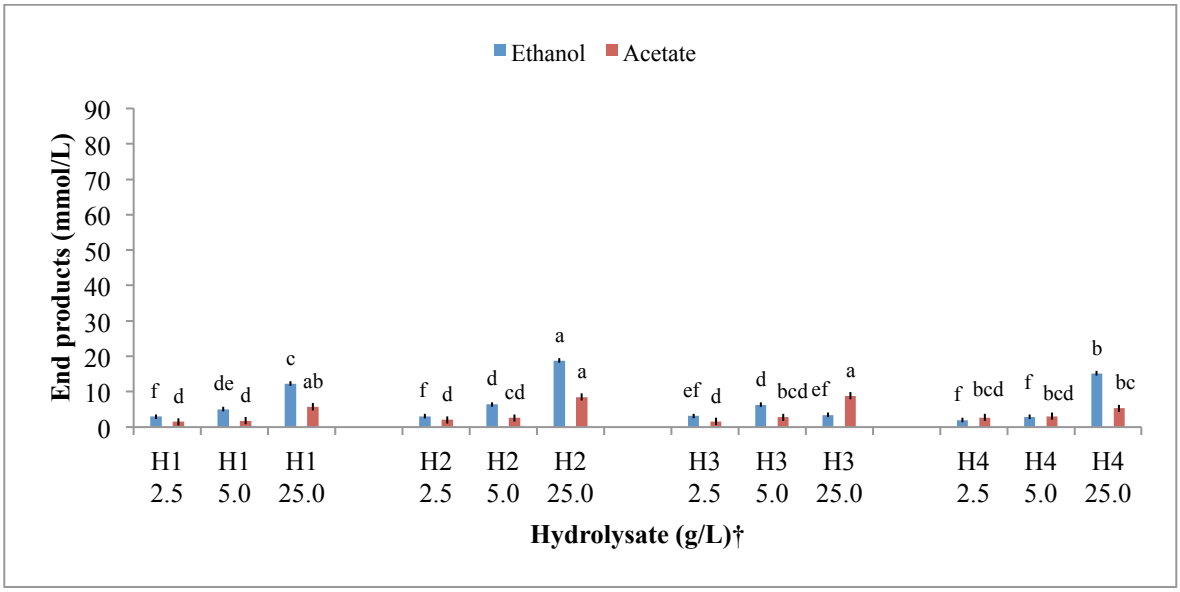

Figure 21. End products formation by *Clostridium thermocellum* on timothy HLs, comparing four harvest times and three HLs concentrations. Means with different letters (abc) within the same end product are significantly different according to ST at 95% confidence level. Bars correspond to SE of mean. †HLs were prepared with weighted samples (DM 0.93).

Table 12 shows the statistical comparison for harvest times across HLs concentration. There was a significant difference for ethanol production for all harvest times except H1 and H4.

Table 12. Harvest time effect on ethanol production by *Clostridium thermocellum* (main effects across HLs concentrations). Means with different letters (abc) are significantly different according to ST at 95% confidence level.

Harvest time		
	Mean ethanol production (mM)	**ST**
H1	6.7	b
H2	9.4	a
H3	4.3	c
H4	6.7	b
Mean	6.8	
SE of mean	0.37	

The figure below (figure 22) illustrates the production of end products by *T. ethanolicus* from the different timothy HLs. The highest ethanol concentration was 49.8 mM, obtained from 25 g/L HLs from H2 (2.1 mM/g DM (control values (growth on YE) subtracted). The HLs with 25 g/L concentrations always showed highest ethanol concentration for each harvest. The highest ethanol yields was obtained from this same HLs, but on 2.5 g/L or 3.5 mM/g DM (control values (growth on YE) subtracted). Acetate concentration was highest for 25 g/L of H2, H3 and H4, 18.3 mM, 18.8 mM and 18.6 mM, respectively. Hydrogen concentration was highest for 25 g/L of H1 (16.4 mmol/L), however, not significantly different from the hydrogen production from 25 g/L of H2 (14.4 mmol/L).

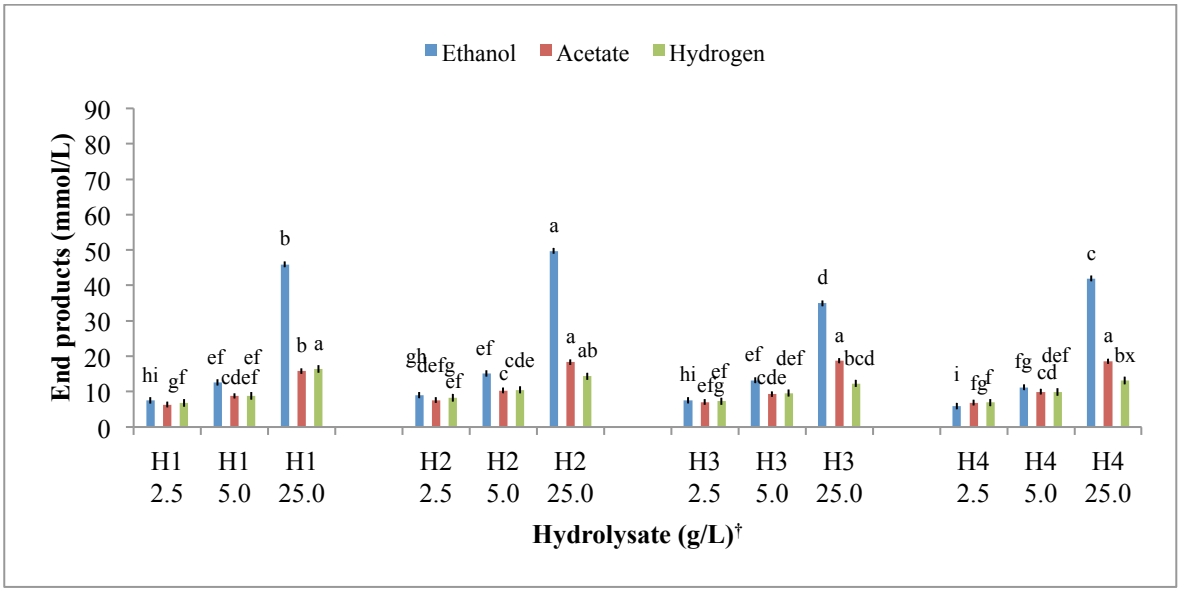

Figure 22. End products formation by *Thermoanaerobacter ethanolicus* on timothy HLs, comparing four harvest times and three HLs concentrations. Means with different letters (abc) within the same end product are significantly different according to ST at 95% confidence level. Bars correspond to SE of mean. †HLs were prepared with weighted samples (DM 0.93).

Table 13 shows the statistical comparison for harvest times and HLs concentration. There was a significant difference for ethanol production for all harvest times except H3 and H4.

Table 13. Harvest time effect on ethanol production by *Thermoanaerobacter ethanolicus* (main effects across HLs concentrations). Means with different letters (abc) are significantly different according to ST at 95% confidence level.

Harvest time		
	Mean ethanol production (mM)	**ST**
H1	22.0	b
H2	24.6	a
H3	18.6	c
H4	19.7	c
Mean	21.2	
SE of mean	0.50	

Figure 23 shows the results for *Thermoanaerobacter* strain J1. By increasing HLs concentration, the higher amounts of ethanol were produced. The highest ethanol concentrations were obtained from HLs concentration of 25 g/L for H1, H2, and H4, 65.8 mM, 71.2 mM and 68.6 mM (2.7, 3.0, 2.8 mM/g DM (control values (growth on YE) subtracted), respectively. Highest ethanol yields were on the 5 g/L concentration of H2 or 4.0 mM/g DM (control values (growth on YE) subtracted). The acetate concentration was highest from 25 g/L of H2 (16.5 mM). The highest hydrogen concentration was from 25 g/L of H1 (10.7 mmol/L).

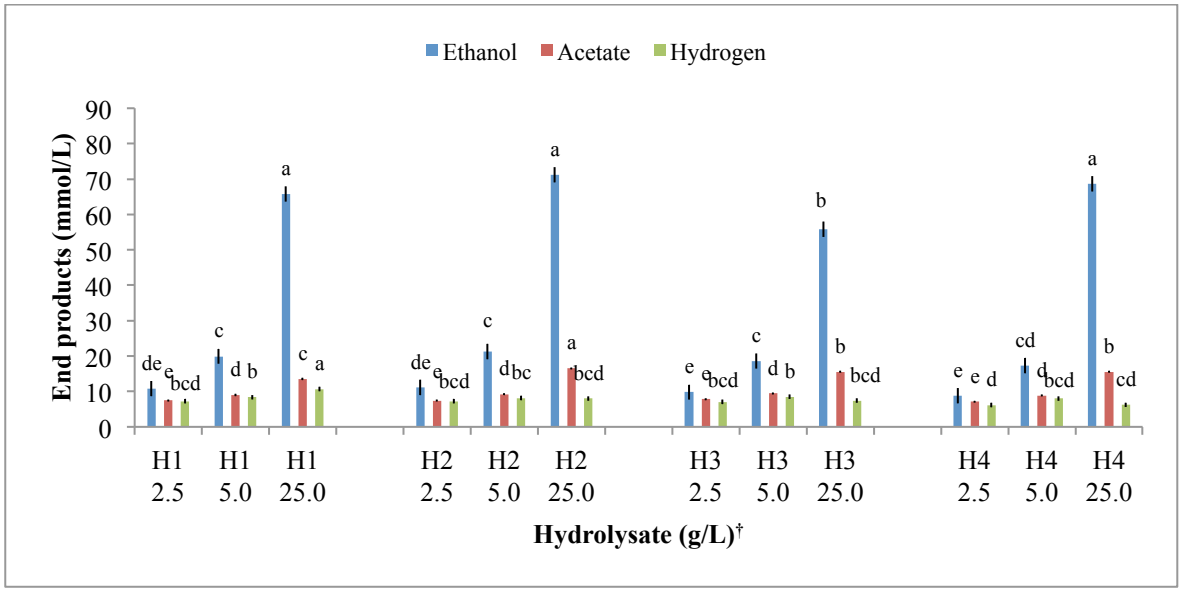

Figure 23. End products formation by *Thermoanaerobacter* strain J1 on timothy HLs, comparing four harvest times and three HLs concentrations. Means with different letters (abc) within the same end product are significantly different according to ST at 95% confidence level. Bars correspond to SE of mean. †HLs were prepared with weighted samples (DM 0.93).

The statistical comparison for harvest times and HLs concentration is shown in table 14. There was a significant difference for ethanol production between the earliest harvest times, H1 and H2 compared to H3, but H4 was not significantly different from the other harvest times.

Table 14. Harvest time effect on ethanol production by *Thermoanaerobacter* strain J1 (main effects across HLs concentrations). Means with different letters (abc) are significantly different according to ST at 95% confidence level.

	Harvest time	
	Mean ethanol production (mM)	**ST**
H1	32.2	a
H2	34.5	a
H3	28.1	b
H4	31.6	ab
Mean	31.6	
SE of mean	1.24	

Figure 24 shows the results for *Z. mobilis* on the different HLs. The ethanol concentration was rather low; the highest value resulted in 15.5 mM for one-day sample of highest concentration (25 g/L HLs) of H2 or 0.6 mM/g DM (control values (growth on YE) subtracted). Highest ethanol yields were however on the 2.5 g/L concentration of H3 or 1.4 mM/g DM (control values (growth on YE) subtracted). The highest acetate concentration was from 25 g/L of H2 (12.1 mM).

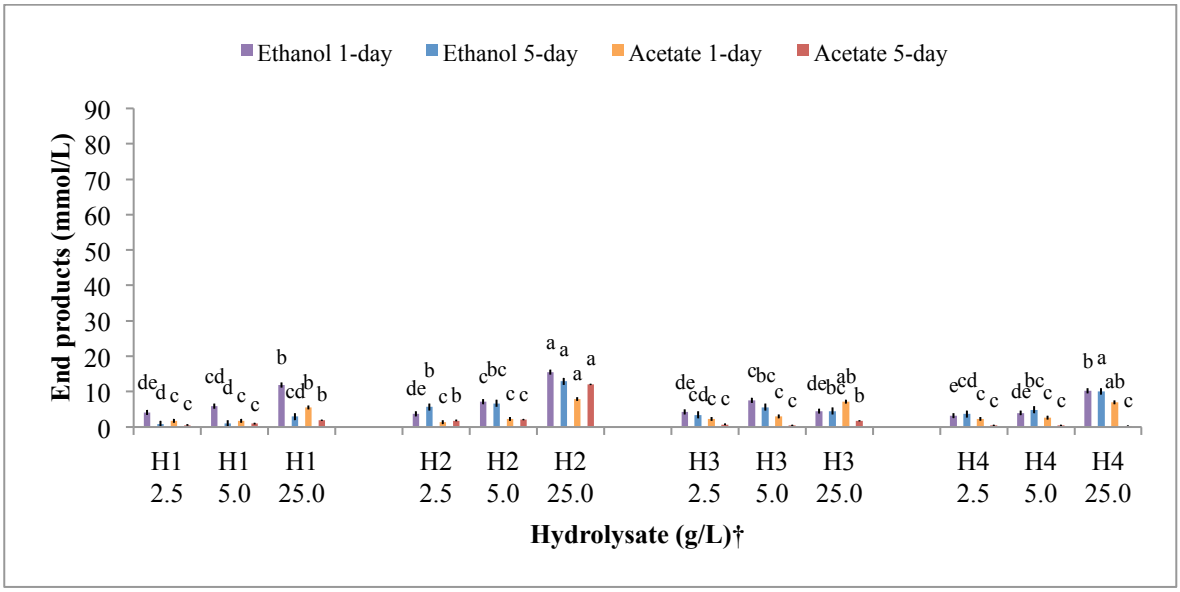

Figure 24. End products formation by *Zymomonas mobilis* on timothy HLs, comparing four harvest times and three HLs concentrations. Means with different letters (abc) within the same end product and day are significantly different according to ST at 95% confidence level. Bars correspond to SE of mean. †HLs were prepared with weighted samples (DM 0.93).

Table 15 shows the statistical comparison for harvest times and HLs concentration. For both one-day and five-day sample, there was a significant difference for ethanol production for all harvest times except between H3 and H4.

Table 15. Harvest time effect on ethanol production by *Zymomonas mobilis* (main effects across HLs concentrations). Means with different letters (abc) are significantly different according to ST at 95% confidence level.

	Harvest time		
		Mean ethanol production (mM)	ST
One-day	H1	7.3	b
	H2	8.8	a
	H3	5.4	c
	H4	5.8	c
	Mean	6.8	
	SE of mean	0.42	
Five-day	H1	1.6	c
	H2	8.4	a
	H3	4.5	b
	H4	6.2	b
	Mean	5.2	
	SE of mean	0.56	

The highest ethanol concentration by *K. marxianus* (figure 25) was obtained from the 25 g/L HLs, for the one-day samples. The highest concentration obtained was 71.3 mM from 25 g/L of H1 or 3.0 mM/g DM (control values (growth on YE) subtracted). The five-day samples resulted in much lower ethanol concentration. Highest ethanol yields were on the 2.5 g/L concentration of H1 or 5.3 mM/g DM (control values (growth on YE) subtracted). Acetate concentration was low for both one-day and five-day samples, except for the 5-day samples on the highest concentration of HLs.

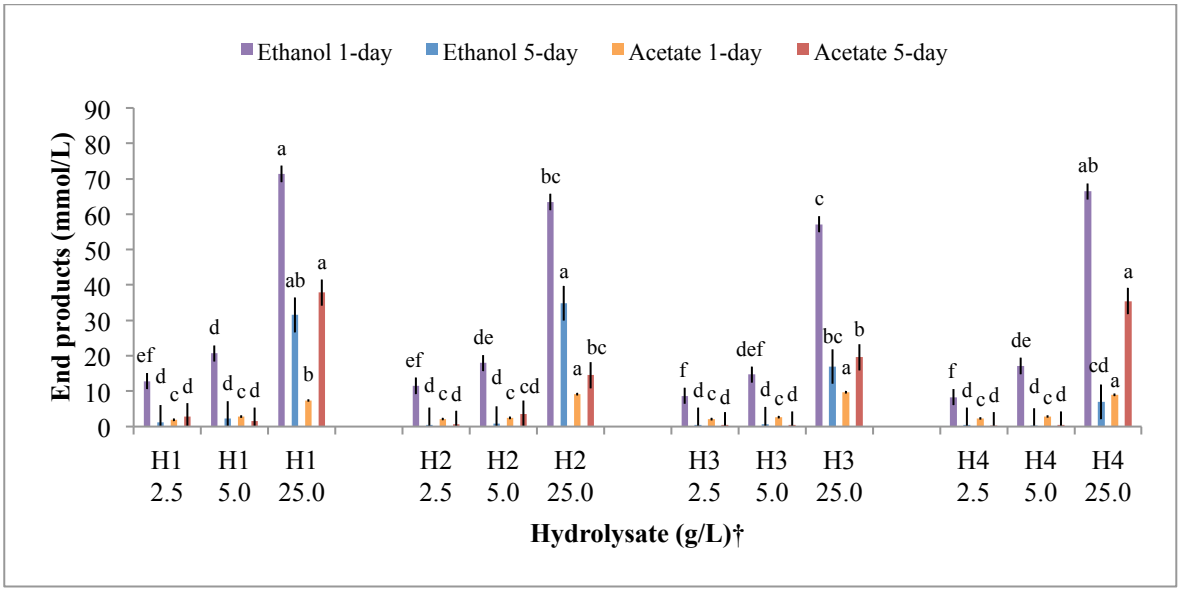

Figure 25. End products formation by *Kluyveromyces marxianus* on timothy HLs, comparing four harvest times and three HLs concentrations. Means with different letters (abc) within the same end product and day are significantly different according to ST at 95% confidence level. Bars correspond to SE of mean. †HLs were prepared with weighted samples (DM 0.93).

The statistical comparison for harvest times and HLs concentration is shown in table 16. For the one-day samples, there was a difference in production between the first two harvest times and H4. There was also a significant difference between H1 and the last two harvests.

Table 16. Harvest time effect on ethanol production by *Kluyveromyces marxianus* (main effects across HLs concentrations). Means with different letters (abc) are significantly different according to ST at 95% confidence level.

		Harvest time	
		Mean ethanol production (mM)	ST
One-day	H1	35.0	a
	H2	31.0	ab
	H3	26.8	bc
	H4	30.6	c
	Mean	30.8	
	SE of mean	1.33	
Five-day	H1	11.7	a
	H2	12.1	a
	H3	6.0	ab
	H4	2.6	b
	Mean	8.1	
	SE of mean	2.82	

The results for *S. cerevisiae* ethanol production from the different HLs is shown in figure 26. The highest concentration was 72.7 mM from 25 g/L HLs of H2 for the one-day sample or 3.1 mM/g DM (control values (growth on YE) subtracted), however there was not a significant difference between H1, H2, and H4 for highest concentration of HLs. Highest ethanol yields were however on the 2.5 g/L concentration of H2 or 5.9 mM/g DM (control values (growth on YE) subtracted). Acetate production was little for most HLs and different concentrations but resulted highest 11.3 mM from 25 g/L of H2. It varied, whether one-day or five-day samples resulted in higher ethanol production.

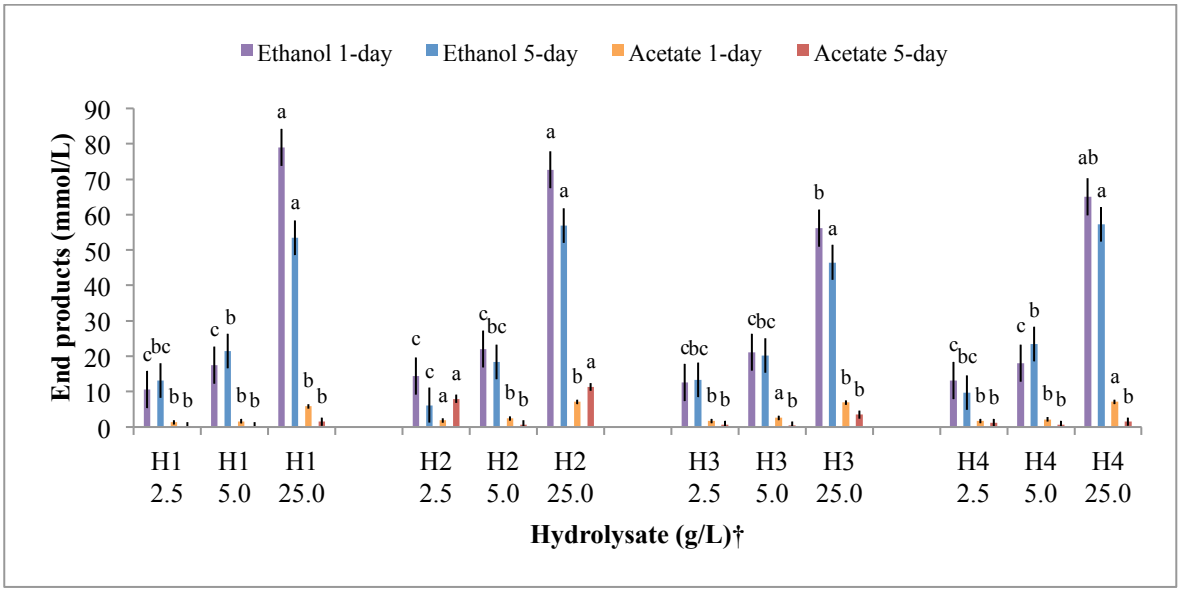

Figure 26. End products formation by *Saccharomyces cerevisiae* on timothy HLs, comparing four harvest times and three HLs concentrations. Means with different letters (abc) within the same end product and day are significantly different according to ST at 95% confidence level. Bars correspond to SE of mean. †HLs were prepared with weighted samples (DM 0.93).

Table 17 shows the statistical comparison for harvest times and HLs concentration. For both one-day and five-day sample, there was not a significant difference for ethanol production between harvest times.

Table 17. Harvest time effect on ethanol production by *Saccharomyces cerevisiae* (main effects across HLs concentrations). Means with different letters (abc) are significantly different according to ST at 95% confidence level.

	Harvest time		
		Mean ethanol production (mM)	ST
One-day	H1	35.7	a
	H2	36.4	a
	H3	30.0	a
	H4	32.1	a
	Mean	33.5	
	SE of mean	3.03	
Five-day	H1	29.4	a
	H2	27.1	a
	H3	26.7	a
	H4	30.2	a
	Mean	28.3	
	SE of mean	2.83	

4.2.6 Ethanol Production from Timothy without Acid/Enzyme Pre-treatment

The results for *C. thermocellum* ethanol production from three different concentrations of timothy without acid/enzyme pre-treatment resulted in no ethanol production and very little acetate production. Therefore no further analysis was performed on this data.

4.2.7 Ethanol Yield and Production Efficiency

The results from the experiments on the different HLs were used to calculate the possible production in litres of ethanol/hectare, using the harvest yield data of the timothy and the ethanol yield. The calculation is only for the four best ethanol-producing organisms, based on one-day samples for the yeasts and 5-day samples for the bacteria. As shown in figure 27, *S. cerevisiae* resulted repeatedly in highest ethanol yield/area on the timothy HLs. The highest ethanol yield/area by *S. cerevisiae* was 2,211 L/ha from 2.5 g/L HLs of H4 and then 2,073 L/ha for same concentration of H3 HLs. *K. marxianus* resulted generally second best, then J1 and *T. ethanolicus*. Interestingly, the lower concentrations of HLs resulted in higher ethanol yield.

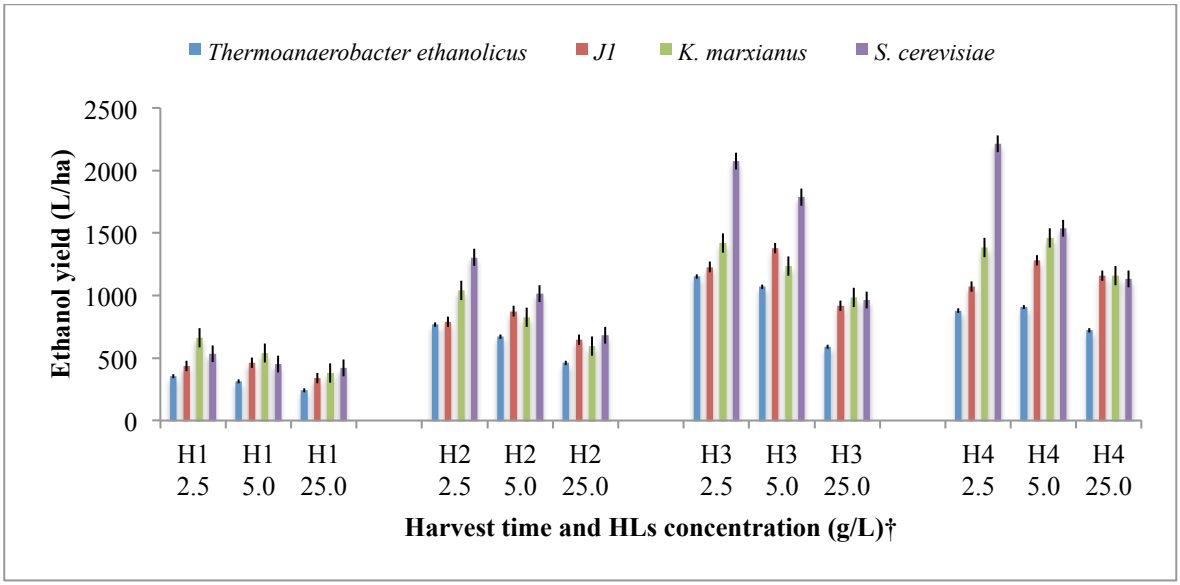

Figure 27. Harvest time and HLs concentration (g/L) effect on ethanol yield (L/ha) by the four best ethanol-producing organisms, for this project. Bars correspond to SE of mean. †HLs were prepared with weighted samples (DM 0.93), but calculations are based on timothy with 100% DM.

Table 18 shows the harvest time effect on ethanol yield/area. There was a significant difference between all harvest times for *T. ethanolicus*. For the other strains, J1, *K.*

marxianus and *S. cerevisiae*, there was a significant difference between all harvest times, except between H3 and H4.

Table 18. Harvest time effect on ethanol yield/area by the four best ethanol-producing strains (main effects across HLs concentrations). Means with different letters (abc) within strains are significantly different according to ST at 95% confidence level.

	T. ethanolicus		**J1**		***K. marxianus***		***S. cerevisiae***	
Harvest time	**Mean ethanol production (L/ha)**	**ST**	**Mean ethanol production (L/ha)**	**ST**	**Mean ethanol production (L/ha)**	**ST**	**Mean ethanol production (L/ha)**	**ST**
H1	304	d	412	c	529	c	470	c
H2	634	c	772	b	822	b	1003	b
H3	935	a	1172	a	1208	a	1604	a
H4	833	b	1166	a	1330	a	1622	a
SE of mean	17.3		41.7		76.8		67.1	

The highest production efficiency was 346 L/t DM from the lowest HLs concentration of H2 by *S. cerevisiae* (figure 28). For the same concentration (2.5 g/L), *S. cerevisiae* for H3 and H4 resulted in 298 and 313 L/t DM timothy, respectively. Interestingly, the latter harvest times are resulting in relatively similar efficiency as the first harvest times.

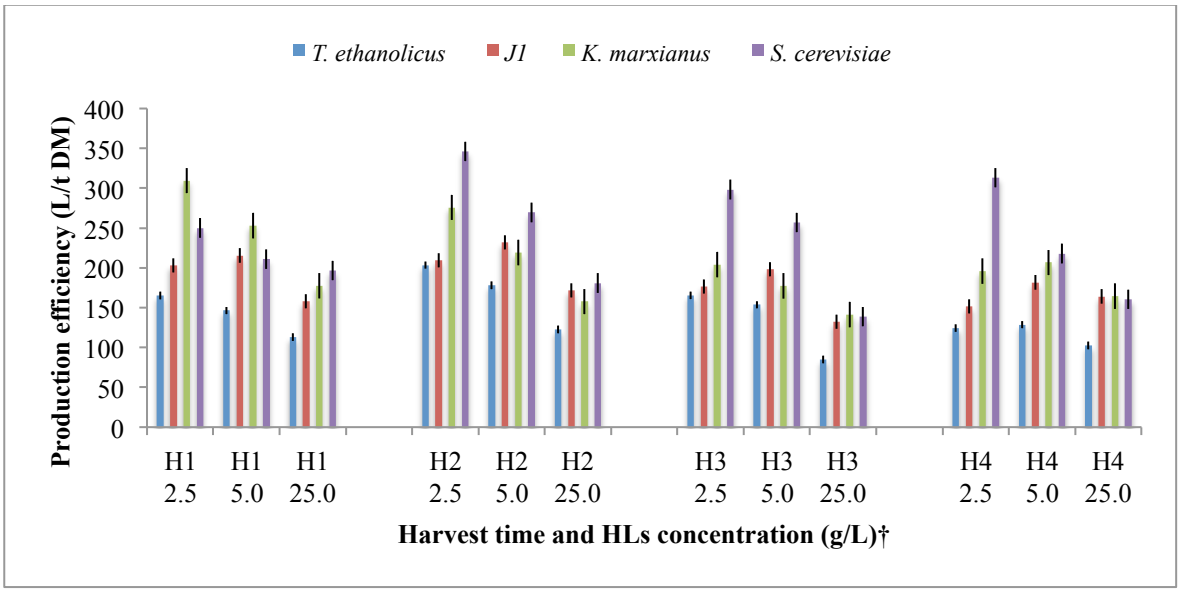

Figure 28. Harvest time and HLs concentration (g/L) effect on ethanol production efficiency (L/t DM timothy) by the four best ethanol-producing organisms, for this project. Bars correspond to SE of mean. †HLs were prepared with weighted samples (DM 0.93) but calculations are based on timothy with 100% DM.

Table 19 shows the harvest time effect on ethanol production efficiency L/t DM. There was a significant difference between all harvest times for *T. ethanolicus,* except between H1 and H3. For J1, H2 was significantly different from H3 and H4. For *K. marxianus*, H1 was significantly different from H3 and H4. For *S. cerevisiae*, there was a significant difference between H1 and H2.

Table 19. Harvest time effect on ethanol production efficiency yield/t DM by the four best ethanol-producing strains (main effects across HLs concentrations). Means with different letters (abc) within strains are significantly different according to ST at 95% confidence level.

	T. ethanolicus		**J1**		***K. marxianus***		***S. cerevisiae***	
Harvest time	**Mean production efficiency (L/t DM)**	**ST**	**Mean production efficiency (L/t DM)**	**ST**	**Mean production efficiency (L/t DM)**	**ST**	**Mean production efficiency (L/t DM)**	**ST**
H1	151	b	206	ab	264	a	234	b
H2	180	a	218	a	233	ab	284	a
H3	144	b	181	b	186	b	247	ab
H4	127	c	177	b	202	b	247	ab
SE of mean	5.1		9.7		17.0		13.1	

4.3 Hydrolysate Residue Analysis

The results for the residue samples are shown in table 18. The samples were analysed for total DM, organic matter, ash, NDF, DMD, N and C. There was not a significant difference between timothy HLs for content of organic matter and ash. There was a significant difference between all HLs for N content and the C/N ratio. The N% of DM was highest for H1 (3.4% of DM) and declined rapidly with maturity and lowest for H4 (1.0% of DM). The C/N ratio was lowest for H1 (15:1) but highest for H4 (49:1). The two latter harvest times resulted in higher NDF% of DM. There was also a significant difference between the two first harvest times and the two latter for C% of DM.

Table 20. Residue samples analysis. Values are shown in % of DM. Means within the same column with different letters (abc) are significantly different according to ST at 95% confidence level.

Harvest time	Organic matter		Ash		NDF		N		C		C/N	
	Mean	ST	Mean	ST	Mean	ST	Mean	ST	Mean	ST	Mean	ST
1	86.0[Q]	a	14.0[Q]	a	18.7	c	3.4	a	50.5	a	14.9	d
2	87.2	a	12.8	a	35.8	b	2.2	b	49.3	a	22.8	c
3	89.1	a	10.9	a	55.0	a	1.2	c	47.7	b	40.1	b
4	87.5	a	12.5	a	55.3	a	0.9	d	46.6	b	49.4	a
Mean	87.4		12.6		41.2		1.9		48.5		31.8	
SE of mean	0.60		0.60		1.55		0.04		0.30		1.71	

[Q] Only based on data for higher concentration of HLs (50g/L).

4.3.1 DMD and Solubility of Timothy Samples

Figure 29 shows the regression between ruminal DMD and solubility of timothy in this project for the two stock concentrations of HLs. The correlation was not statistically significant (ST at 95% confidence level).

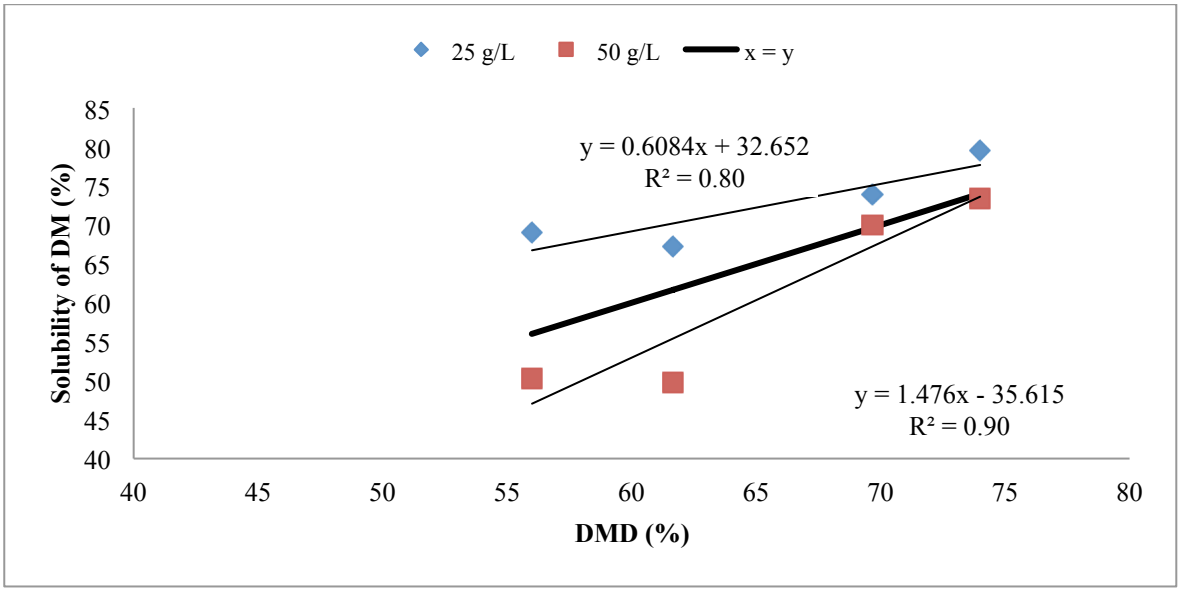

Figure 29. The correlation between DMD and solubility of timothy DM, for the two HLs concentrations, 25 g/L (P=0.1059) and 50 g/L (P=0.0536), for the four harvest times of timothy.

4.4 Results Summary

The results show that the organisms used in this study are very different and selective when it comes to single substrate breakdown for ethanol production. Similarly, these organisms are very different in their capability to utilize lignocellulosic substrate like

timothy for ethanol production. Different harvest time of timothy, with prescribed pre-treatments, did have small but significant effect on ethanol yield. The next chapter, therefore moves on to discuss possible reasons and understanding of these findings in connection with ethanol production efficiency, DM yield and DM composition as well as other agronomic questions.

5. Discussion

5.1 Ethanol Production from Simple Substrates

The results from this part of the study showed how each organism's growth was on several simple substrates found in lignocellulosic biomass. The ethanol yield observed in this project is compared to maximum ethanol yield from literature in table 19. The ethanol yield by the yeasts and *Thermoanaerobacter* strain J1 were close to theoretical maximum but the ethanol yields for other strains were rather far from theoretical yields. This might be explained by the medium used; that it was not suitable for all strains.

Table 21. Comparison of ethanol yield obtained from glucose and avicel in this project and maximum ethanol yield observed in previous studies.

Strain	Substrate	Ethanol yield (mol/mol)		Reference
		This project	Maximum ethanol yield observed	
Clostridium thermocellum	Avicel	0.83[Q]	1.3	(Rani et al., 1997)
Thermoanaerobacter ethanolicus	Glucose	0.6	1.9	(Wiegel & Ljungdahl, 1981)
Thermoanaerobacter strain J1	Glucose	1.7	1.7	(Jessen & Orlygsson, 2012)
Zymomonas mobilis 5-day	Glucose	0.3	1.9	(Bai, Anderson, & Moo-Young, 2008)
Kluyveromyces marxianus 1-day	Glucose	1.7	1.9	(Fonseca, et al., 2008)
Saccharomyces cerevisiae 5-day	Glucose	1.7	1.9	(Bai et al., 2008)

[Q] Glucose equivalent

In present study, *Thermoanaerobacter* strain J1 outperformed *T. ethanolicus*, on all stubstrates except glucose. J1 has been shown to utilize a more diverse range of substrates than *T. ethanolicus* (Jessen, 2013), but this could also be related to the medium used, it could be more ideal for J1 since the strain was isolated on it. As shown in figure 14 and 15, J1 produced ethanol concentrations up to 96.7 mM (48.4 mM/g) from starch as compared to *T. ethanolicus*, which produced 30.8 mM (15.4 mM/g). Theoretical yields of glucose hydrolysis from 2 g/L of starch is 111 mM, and the strain is thus producing 1.02 mol ethanol from glucose equivalent, assuming all starch is hydrolysed to glucose.

Interestingly, the ethanol concentration is lower for five-day samples compared to one-day samples for *K. marxianus.* The other yeast, *S. cerevisiae* showed different results, higher ethanol concentration in five-day samples compared to one-day samples. It seems that after certain time, *K. marxianus* starts to consume ethanol. It is known that oxygen availability is very important for the growth and ethanol production of yeasts. However, too much oxygen supply could lead to excessive yeast growth that may reduce the ethanol yield as carbon is shifted to the production of cell mass (Guimaraes et al., 2010). In this experiment, *K. marxianus* was grown under aerobic conditions without shaking. It is also known that yeasts grown in aerobic batch cultures become oxygen limited in the presence of a high initial carbon substrate (Castrillo et al., 1996). It has been shown that *K. marxianus* indeed degrades ethanol under aerobic conditions at elevated temperatures under shaking conditions when grown on galactose (Rodrussamee et al., 2011).

It would be interesting to investigate further the effect of oxygen supply for *K. marxianus*, grown on lignocellulosic biomass hydrolysates. Additionally it would be important to take samples frequently during incubation, with different oxygen supply levels.

Of the substrates tested *Clostridium thermocellum* is known to degrade both avicel and cellobiose. Therefore, it is surprising that in present investigation the strain only degraded avicel (figure 13). *Thermoanaerobacter ethanolicus* and *Thermoanaerobacter* J1 are both known to degrade various sugars but not cellulose, which is in correlation with the results obtained in present investigation (Wieglel & Ljungdahl, 1981; Jessen & Orlygsson, 2012). Theoretical yields of ethanol from glucose for *Z. mobilis* are much higher than obtained in present study. This could be due to difference in medium used in present study as compared with optimum yields obtained earlier.

5.2 Ethanol Production from Timothy

5.2.1 Timothy Samples

The timothy DM yield in this study is consistent with many other Icelandic studies with timothy and can therefore be used as the base for yield in agronomic scenarios on ethanol production.

Timothy Analysis

There was not a statistically significant correlation between ruminal DMD and DM solubility in this study, even though the trend appears to be clear (figure 28). The conclusion is that DMD cannot be used to estimate the potential solubility of timothy prepared for HLs for ethanol production. It should be noted however that the data set that this is based on was very limited.

5.2.2 Ethanol Production from Whatman Paper

The results for mean ethanol production from WP were in correlation with both the results on simple substrates and timothy HLs. The strains that produced highest yield from the WP had also highest yield from the simple substrates and timothy HLs. However, ethanol yield from experiments with WP was abnormally low and suggests that the enzymatic hydrolysis has not fully succeeded. This can for example be seen when investigating the ethanol values obtained from this substrate by *S. cerevisiae*. Assuming a 100% hydrolysis of 2.5, 5.0 and 25 g/L of WP should lead to production of 15.4, 30.8 and 154 mM of glucose, respectively. This glucose should in turn yield 29.3, 58.6 and 292.6 mM of ethanol (assuming 1.9 mol ethanol yields). The yields obtained were however much lower, or 13.3, 24.1 and 81.4 mM of ethanol (45%, 41% and 28%), respectively. The reason for this inefficiency of hydrolyses is unclear.

5.2.3 Ethanol Production from Timothy Hydrolysates

The first question in this study sought to determine the effect of the harvest time or the growth stage of the timothy on the production of ethanol (mM). In many cases there was not a significant difference of ethanol yield (mM) for different harvest time and therefore, resulting in similar ethanol production efficiency. The amount of ethanol produced is therefore mainly dependant on the timothy yield for each harvest.

In present study, J1 produced 11.1 mM of ethanol from the 2.5 g/L H2 timothy HLs and 21.2 mM from H2 HLs at 5 g/L concentration, resulting in 3.6 and 4.0 mM/g DM timothy (control values (growth on YE) subtracted), respectively, which is slightly lower but consistent with the previous results of similar pre-treated timothy by Jessen and Orlygsson (2012). Their results showed that J1 produced 11.7 mM ethanol from HLs concentration of 2.5 g/L and 21.1 mM from 5 g/L HLs, resulting in ethanol yield of 4.7 and 4.2 mM/g timothy, respectively.

Orlygsson (2013) investigated ethanol production from timothy by *Thermoanaerobacter* strain B2. The timothy was a primary cut, mowed from well-fertilized field at early boot stage. His results showed that with acid/enzyme pre-treatment and at timothy HLs concentration of 4.5 g/L, B2 produced 4.2 mM ethanol/g timothy.

Ethanol Yield and Production Efficiency

The second aim of this research was to find which organisms used showed the best result, with the best production efficiency (L/t DM timothy) and highest ethanol yield/area (L/ha). Ethanol yields for *S. cerevisiae* (figure 28) resulted repeatedly in highest ethanol yield/area on the timothy HLs. The highest ethanol yield/area by *S. cerevisiae* was 2,211 L/ha from 2.5 g/L HLs of H4 and then 2,073 L/ha for same concentration of H3 HLs. Ethanol yields for *K. marxianus* generally were second best, then J1 and *T. ethanolicus*. Interestingly, the lower concentrations of HLs resulted in higher ethanol yield. This could be explained by substrate or end product inhibition, which has been extensively reported for thermophiles (Almarsdottir et al., 2012; Brynjarsdottir et al., 2013; Brynjarsdottir et al., 2012) during increased substrate loadings. The latter harvest times are resulting in much higher ethanol yield, which is related to higher timothy DM yields.

The difference in the ethanol production efficiency (L/t DM of timothy) is much lower than expected (figure 29), especially when compared to the change in timothy ruminal DMD, as mentioned in the literature review. It is possible that this might be relevant to the high N concentration and low C/N ratio of early harvested timothy samples. On the other hand as can be seen in table 9, the initial sugar content is about the same irrespective of harvest time.

5.2.4 Initial Sugar Concentration and Degradation

As discussed earlier, it seems that the hydrolysis did not fully succeed for WP, however it is not known whether the same applies for the timothy samples. However, these results showed slightly less initial sugar concentration of HLs compared to previous results from experiments on similar timothy samples by Jessen (2013). Ethanol yields, in present study, from sugars available in the HLs showed good results. As shown in table 9, the highest theoretical ethanol yield from the timothy HLs was from H4, 5.5 mM/g timothy (control values (growth on YE) subtracted), for that harvest time, *S. cerevisiae* produced 5.4 mM/g DM timothy (figure 26) or 98% of theoretical yields from released sugars in timothy HLs.

The proportion of sugar residues was not affected by harvest time or HLs concentration with the exception of *T. ethanolicus*, like mentioned in the results section. Given these data, neither the harvest time nor HLs concentration has effect on the proportion of sugar residue. The sugar degradation (-7%) of *C. thermocellum* (table 10) was significantly different from degradation by other strains. *C. thermocellum* is known to degrade crystalline cellulose using a complex set of hydrolytic enzymes, called cellulosome (Scully & Orlygsson, 2014). In present study, the sugars concentration was higher after fermentation by *C. thermocellum*, which suggests that the bacterium utilized the cellulose and increased the sugars availability.

5.2.5 Ethanol Production without Acid/Enzyme Pre-treatment

The experiment with *C. thermocellum* grown on timothy samples without acid/enzyme pre-treatment resulted in no ethanol production. The only pre-treatment used for the samples in this experiment was grinding, as was performed for HLs preparation.

Possible explanation for these results could be too high concentration of timothy sample. There could be a great cost benefit if it would be possible to obtain similar ethanol yield from untreated lignocellulosic biomass, as from pre-treated lignocellulosic biomass. As mentioned in the literature review, the pre-treatment of the biomass is often the most costly part of the production. It would be interesting to set out experiments including lower concentration of timothy and see if that would result in more ethanol production. Additionally, it would be interesting to set out experiments with co-fermentations including *C. thermocellum* on timothy HLs.

5.3 Timothy for Biomass Production

As mentioned in the literature review, timothy is an important forage grass in Iceland and has good; yield ability, feed quality, palatability and persistence (Helgadottir & Sveinsson, 2006; Sveinsson, 2001). These qualities make the timothy interesting for biomass production in Iceland. What is surprising about the results from this experiment is how little the efficiency varied between harvest dates and how good the efficiency is for the latter harvest dates, as shown on figure 29. This was unexpected, given the results from earlier studies on the use of timothy as feed that have shown a rapid decline of digestible matter with increased maturity of the grass (I. Bjornsson, 2000; Olafsson, 1979;

Thorvaldsson, 2006). These results are interesting and overall positive for ethanol production from timothy in Iceland. There are many advantages to harvest timothy mid-summer or later.

Results from weekly sampling of primary cut of timothy for yield comparison during the summers of 1979 and 1980 showed that a maximum yield of timothy, 9.2 t DM/ha and 10.4 t DM/ha was obtained when harvested August 28th and August 12th, respectively. These results can be regarded to be timothy maximum yield of primary cut in Iceland (H. Bjornsson, 1987; H. Bjornsson et al., 2004). The total timothy yield from experiments on mowing height, where timothy was harvested twice during the summer, gave good results for total yield up to 8.62 t DM/ha, when primary harvest performed in late July and second cut in late August or early September (Sveinsson, 2003). Another experiment gave higher total timothy yield for two harvests or 9.53 t DM/ha, based on three-year average 1991-1993. The first cut resulted in yield of 8.85 t DM/ha and the second harvest in 0.68 t DM/ha, when harvested June 26th and July 29th, respectively (Brynjolfsson, 1994).

Timothy is vulnerable for mowing during stem elongation (Mcelroy & Kunelius, 1995). But if timothy primary cut is made after anthesis, it is possible to maintain good cover for long time (Hermannsson & Helgadottir, 1991). The timing of the primary harvest of timothy determines its persistence and longevity (Sveinsson, 2003).

If timothy is harvested after anthesis or later it is not necessary to mow the fields more than once. That should result in lower cost and better production efficiency, even though two harvests would result in similar total yield that would have better qualities for the ethanol production. The cost of the second harvest, in use of machinery, fertiliser and work are probably higher. Additionally, double harvest losses on the field would be expected when harvested twice and the weather conditions for haymaking are often less favourable for the timing of second harvest (Brynjolfsson, 1994).

This experiment used dried primary cut of timothy but it would be interesting to set out experiments using second harvest of timothy. Moreover, since wilted round bale silage (haylage) is the main curing method in Iceland (Helgadottir & Sveinsson, 2006), it is suggested that future experiments on ethanol production from timothy or other grasses

should use haylage or silage as substrates. It is important to find out how different storage methods and storage time will affect the overall ethanol production.

5.4 Possible Biofuel Production in Iceland

There has been some discussion on the production of biofuels in Iceland, about the possibilities to use agricultural waste and also to produce biomass specifically grown for biofuel production purposes (H. Bjornsson, 2007; Snaebjornsson et al., 2010; Sveinsson & Hermannsson, 2010).

Sveinsson and Hermannsson (2010) reviewed many options and possibilities for biofuel production in Iceland. They considered different feedstock types, storage methods and possible locations, both for biomass production and for the fuel processing. One option they reviewed was ethanol production from perennial grasses and mention that timothy would be a likely choice. They consider the effect of early and late harvest, as reviewed in this thesis, and concluded for the perennial grasses that it would be important that the biomass production would be in rather large and in continuity, close to ethanol producing facility.

H. Bjornsson (2006) considers whether biofuel production from biomass in Iceland is a valid option. His main concerns about the issue is the size of arable land needed for large-scale biomass production and also because other available low-cost energy resources like electricity that can be used for example to produce hydrogen.

The method used in this project for ethanol production is relatively new and much more data and cost analysis is needed before it is possible to conclude, whether ethanol production from timothy or other biomass types are a viable option in Iceland.

5.4.1 GDD in Iceland

It is important to realize the limitations for crop production in Iceland. Although there has been progress in plant breeding and new cultivars, the production must be based on crops that will give an acceptable yield with certain guarantee. The best areas for crop production in Iceland have a mean of 1300 GDD. This is a major limiting factor for crop production and as reviewed in the literature, a crop that needs the mean number of GDD to grow would only give harvest every other year because of variation between summer seasons.

Additionally, if 100 GDD are added to the crop requirement it should be possible to get 4 harvests out of every 5 growing seasons (Hermannsson, 2001).

In this experiment, total number of GDD was 1290 from May until September. As mentioned in the results, the total GDD until harvest was 265, 410, 703 and 1,081 for the harvest dates of June 4th, June 16th, July 10th and August 11th, respectively.

As the results of total GDD show, Möðruvellir in Hörgárdalur would be categorized in the number one growing cultivation zone in Iceland, as mentioned in the literature review. Therefore, it can be assumed that the samples used for this experiment are of good quality.

5.4.2 Arable Land

The third question in this research was to evaluate how much ethanol can be produced in Iceland using the best results from experiments performed. The results from this experiment explain how much ethanol can be produced per hectare but the area usable for that sort of production is unknown. It is surprising how little information is available on the subject, given how valuable land resources are. There has been some discussion on potential arable land in Iceland and few attempts have been made to find out the size of potential arable land. The conclusions from these projects, as listed in the literature review, have varied greatly depending on the preconditions given for each estimation and method used. Sveinsson and Hermannsson (2010) concluded potential arable land for large-scale biomass production to be about 420 km^2, Áslaug Helgadóttir and Jónatan Hermannsson estimation for the Icelandic Ministry of Fisheries and Agriculture resulted in total of 6,000 km^2 (Snaebjornsson et al., 2010) and Traustason and Gisladottir (2009) estimation resulted in 6,150 km^2.

Possible Ethanol Production from Timothy

It was determined to use Sveinsson and Hermannsson (2010) estimation of potential arable land for calculations on how much ethanol can be produced in Iceland. That estimation on potential arable land, focused on area fit for large-scale biomass production that is still not cultivated and therefore, the most relevant estimation for this project. They concluded that potential arable land in Iceland, not in use and suitable for large-scale biomass production was approximately 420 km^2. Their criterion is further listed in the literature review.

The results with highest ethanol yield (H4, 2.5 g/L by *S. cerevisiae*) from this project showed that it is possible to produce approximately 2,211 L/ha. That calculates to about 92.9 million L of ethanol that would be possible to produce in Iceland if the 420 km^2 would be used for the production with good outcome. The total domestic gasoline use (not including aviation gasoline) was approximately 189 million litres in 2011 (Orkuspárnefnd, 2012). Given that potential bioethanol production in Iceland would be 92.9 million L or 61.9 million litres of gasoline-equivalent, which calculates to approximately 33% of domestically gasoline use (not including aviation gasoline). The potential production of bioethanol could be used instead of imported gasoline or in mixture with gasoline (gasohol). The possible amount of ethanol should be sufficient for low ethanol blends (mixture by volume), for example fuel mixture of 10% ethanol and 90% gasoline, commonly known as E10. The E10 gasohol is an interesting option, since no special modification of engine or fuel system of modern vehicles is needed (Balat, 2009). Also, if the ethanol would be used as gasohol, it would not affect the infrastructure installed for transporting or the use of oil products (Szklo et al., 2007). Additionally, it could be beneficial for the Icelandic economy, to produce part of the fuel domestically used.

It is important to consider that the hay field at Möðruvellir in Hörgárdalur is a first class farmland for Icelandic conditions and in growing cultivation zone one, as listed in the literature review. Therefore, it is likely that not all the potential arable land, 420 km^2 would return the same timothy yield as the field in Möðruvellir. However, most farmland in Iceland is below 200 m above sea level and as shown in Hermannsson (2001) estimation for growing cultivated zones in Iceland, all farmland below 200 MASL should have at least mean 1040 GDD. Therefore, it should be expected to get acceptable timothy yield from these 420 km^2, like the best ethanol yield results from this experiment showed, harvest times H3 and H4 had 703 and 1081 GDD until harvest, respectively.

This estimation does not taken into account arable land that is in use. But there might be an opportunity both for farmers and future ethanol producers to use excess feed for this production or feed that is left over from the winter before. Also, farmers could plan their crop production with the additional production of biomass for bioenergy as a side product to other agricultural production. However, it would be risky for the ethanol production to be dependent upon surplus feedstock or leftovers from feed production. It is likely that the

amount of biomass available could fluctuate greatly between years and it is likely that extra or left over feed would be of lower quality for ethanol production.

5.5 Hydrolysate Residue

A possible use for the residue formed in the production process of ethanol from timothy was additionally considered in this study.

5.5.1 Amount of Hydrolysate Residue formed and Nitrogen Content

The current study found that there was a significant difference of N content between harvest times. As expected, the N content was highest (3.4%) for the earliest harvest date and lowest (1.0%) for the last one. The results also show a significant difference in C/N ratio, ranging from 14.9:1 for residues from the first harvest time to 49.4:1 for the residue for the last harvest time.

It is important to consider the amount of residue that forms as a by-product of the ethanol production. As shown in results, the solubility of the biomass varied from 50% to 79%. As expected, the analysis of the residue shows that the solubility of the timothy decreases with increased maturity. There are more soluble compounds, including carbohydrates, in the timothy samples from earlier harvest times compared to the later ones. However, this is not resulting in much higher ethanol production efficiency (L/t DM of timothy), like one would expect. The higher concentrations of HLs resulted in less efficiency. Too high HLs loading could cause substrate inhibition by the fermenting organism.

The data for the HLs solubility shows, that for every kg DM of timothy used for fermentation the residue will range from 21% to 50%. For one litre of ethanol produced (using the best yielding results from this experiment, H4, concentration 2.5 g/L by *S. cerevisiae*), using 3.2 kg DM of timothy with solubility of 69%, there will be 1.0 kg of residue. That residue would contain about 9.9 mg of N (H4, 2.5 g/L, N 1.0% DM of HLs residue).

HLs Residue Summary

If ethanol would be produced at a large-scale facility using this method, the large amount of residue produced is unavoidable. The residue formed during the process would be

needed to dispose in some way. These results suggest that since there is some amount of N in the residue, plus the ash it might be possible to use as fertiliser or for soil amendment. Maybe it would also be possible to use it as burning material for other energy production or as material for methane production. This is an important issue for future research, to develop a full picture of the environmental effect of ethanol production from lignocellulosic biomass and the residue formed in the process. The fertiliser value of this residue can only be determined with experimentation.

6. Conclusion

The results of this research indicate that different harvest time of timothy had a significant effect on ethanol yield (mM of timothy HLs). However, the difference was not significant for all harvest times or for all strains. But there was often a difference between earlier harvest dates and the latter ones. The ethanol production efficiency varied less between harvest times, than expected, especially considering the harvest time effect on timothy ruminal DMD. The first harvest time was usually significantly different from the rest. However, when ethanol yields were calculated on the basis of area for the best ethanol-producing strains, there was, in most cases, a significant difference in ethanol yield from timothy HLs between harvest times prior to mid-heading (H1 and H2) and after full-heading (H3 and H4). This was mainly because of higher timothy primary yield/area for the latter harvest dates. It is important to keep in mind that this is only calculated for the primary cut of timothy. For early harvest times of timothy, most fields would likely be harvested twice, which would increase the potential ethanol production on area basis. However, there are many advantages of only one cut per growing season of timothy, like good yield and persistence, high production efficiency and less production cost. The timothy yield in this project was consistence with many other Icelandic studies with timothy and can therefore be used as the base for yield in agronomic scenarios on ethanol production.

S. cerevisiae resulted as the best ethanol-producing strain in this project. *S. cerevisiae* had the highest ethanol production efficiency, 346 L/t DM of H2 timothy, at the lowest concentration (2.5 g/L) of HLs used. For the same concentration (2.5 g/L), *S. cerevisiae* for H3 and H4 resulted in 298 and 313 L/t DM of timothy, respectively. This calculates to ethanol yield/area by *S. cerevisiae* 1,304 L/ha from H2, 2,073 L/ha from H3 and highest 2,211 L/ha for H4. Given that the potential arable land for large-scale biomass production in Iceland is approximately 420 km^2, it should be possible to produce about 92.9 million L of ethanol by this method.

This project revealed that the HLs solubility ranged from 50% to 79%. This means that for one litre of produced ethanol residue (using the best yielding results from this experiment, H4, concentration 2.5 g/L by *S. cerevisiae*), using 3.2 kg DM of timothy with solubility of

69%, there will be 1.0 kg of residue. That residue would contain about 9.9 mg of N (H4, 2.5 g/L, N 1.0% DM of HLs residue).

The method used in this project for ethanol production is relatively new. Much more data and cost analysis is needed before it is possible to conclude whether ethanol production from timothy or other biomass types are a viable option in Iceland. It is suggested that future research on ethanol production from timothy or other grasses should use haylage or silage as substrates. It is important to find out how different storage methods and storage time will affect the overall ethanol production. Another possible area of future research would be to develop a full picture of the environmental effect of ethanol production from timothy and the residue formed in the process. The fertiliser value of this residue can only be determined with experimentation.

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