Eesti Maaülikool
Estonian University of Life Sciences

FACTORS AFFECTING BIOCHEMICAL COMPOSITION OF LIGNOCELLULOSIC BIOMASS AND ITS EFFECT ON SELECTION OF PRETREATMENT METHOD AND ON BIOETHANOL PRODUCTION POTENTIAL

LIGNOTSELLULOOSSE BIOMASSI BIOKEEMILIST KOOSTIST MÕJUTAVAD TEGURID NING BIOKEEMILISE KOOSTISE MÕJU EELTÖÖTLUSMEETODI VALIKULE JA BIOETANOOLI TOOTLIKKUSELE

MARTI TUTT

A Thesis
for applying for the degree of Doctor of Philosophy
in Bioenergetics

Väitekiri
Filosoofiadoktori kraadi taotlemiseks bioenergeetika erialal

Tartu 2015

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**Doctoral Theses of the
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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The present thesis is based on the following research papers:

I.   Kikas, Timo; Tutt, Marti; Raud, Merlin; Alaru, Maarika; Lauk, Ruth; Olt, Jüri (2014). Basis of Energy Crop Selection for Biofuel Production: Cellulose vs. Lignin. International Journal of Green Energy, xxx - xxx. (in press).

II.   Tutt, M.; Kikas, T.; Kahr, H.; Pointner, M.; Kuttner, P.; Olt, J. (2014). Using steam explosion pretreatment method for bioethanol production from floodplain meadow hay. Agronomy Research, 12(2), 417 - 424.

III.   Tutt, M.; Kikas, T.; Olt, J. (2013). Influence of harvesting time on biochemical composition and glucose yield from hemp. Agronomy Research, 11(1), 215 - 220.

IV.   Tutt, M.; Kikas, T.; Olt, J. (2012). Influence of different pretreatment methods on bioethanol production from wheat straw. Agronomy Research, 10(1), 269 - 276.

V.   Tutt, M.; Raud, M.; Kahr, H.; Pointner, M.; Kikas, T.; Olt, J. (2015). Nitrogen explosion pretreatment of lignocellulosic material for bioethanol production. Energy Sources, Part A: Recovery, Utilization, and Environmental Effects, xxx – xxx, (in press).

The contribution of the author to the papers for publication

Paper	Study of design	Data collection	Data analysis	Manuscript preparation
I	JO, **MT**, TK, MA	**MT**, MA, TK, RL	**MT**, MA, TK, MR, RL	TK, **MT**, MA, MR, JO
II	**MT**, TK, HK, JO	**MT**, MP, PK, TK	**MT**, TK, MP	**MT**, TK, JO
III	**MT**, TK, JO	**MT**	**MT**, TK	**MT**, TK, JO
IV	**MT**, TK, JO	**MT**	**MT**, TK	**MT**, TK, JO
V	TK, **MT**, MR, JO, HK, MP	**MT**, TK, MR, MP	**MT**, TK, MR	**MT**, TK, MR, JO

MT – Marti Tutt; HK – Heike Kahr; JO – Jüri Olt; MA – Maarika Alaru; MP – Manuel Pointner; MR – Merlin Raud; PK – Paul Kuttner; RL – Ruth Lauk; TK – Timo Kikas.

ABBREVIATIONS AND SYMBOLS

Abbreviations

AFEX	Ammonia fibre explosion
BY	Biomass yield
CEL	Cellulose
CHP	Combined heat and power
DM	Dry matter content
EBY	Experimental bioethanol yield
EBYA	Experimental bioethanol yield per area
EC	European Commission
FE	Fermentation efficiency
FY	Fermentation yield
GHG	Greenhouse gas
GY	Glucose yield
HCEL	Hemicellulose
HE	Hydrolysis efficiency
HMF	Hydroxymethylfurfural
HPLC	High performance liquid chromatography
LIG	Lignin
NE	Nitrogen explosion
OE	Overall efficiency
RI	Refractive index
SE	Steam explosion

SEM	Scanning electron microscope
TBP	Theoretical bioethanol potential
TBPA	Theoretical bioethanol potential per area
toe	Tonne of oil equivalent
UV	Ultraviolet

Symbols

B_{by}	experimentally measured biomass yield, $g\ m^{-2}$
B_{tbp}	theoretical bioethanol potential, $g\ kg^{-1}$
B_{tbpa}	theoretical bioethanol potential, $g\ m^{-2}$
C_{cel}	cellulose content, %
C_{DM}	dry matter content, %
E_{fe}	fermentation efficiency, %
E_{he}	hydrolysis efficiency, %
E_{oe}	overall bioethanol production efficiency, %
f	frequency, Hz
k_i	inhibition constant
K_m	kinetic constant that equals to substrate concentration, at which half of the maximum speed of reaction is achieved
m_{eth}	theoretical quantity of ethanol calculated from experimentally measured quantity of glucose, g
$m_{E.eth}$	experimentally measured quantity of ethanol, g
$m_{E.gluc}$	experimentally measured quantity of glucose, g
m_{gluc}	theoretical quantity of glucose, g

m_{sample}	quantity of sample, g
n	number of values in data set
p	statistical significance
P	pressure, bar
P_c	product concentration
P_e	electric power, W
S	substrate concentration
t	time
T	temperature, °C
U	voltage, V
V	speed of enzyme reaction,
V_{max}	maximum speed of enzyme reaction
x	value of the data set
Y_{eby}	experimentally measured bioethanol yield, g kg^{-1}
Y_{ebya}	experimentally measured bioethanol yield, g m^{-2}
$\bar{x}$	statistical mean of the data set
σ	standard deviation

1. INTRODUCTION

Growing environmental concerns about increasing emissions of greenhouse gases, constant price fluctuations in the fuel market, together with political instability in some of the main oil exporting regions have prompted a continuous move towards the use of more evenly distributed clean and renewable fuels in the world (Wei et al., 2014). For example the European Union has set a target of reaching 20% share of renewable energy in the overall energy production for the year 2020 (European Commission, 2009). This also includes that 10% of fuel used in transportation sector has to be produced from renewable resources. Among many renewable energy alternatives for transportation fuels, liquid biofuels and biomethane are considered as the most favourable choices, because these fuels are renewable, biodegradable and can be produced from a wide range of raw materials (Nigam and Singh, 2011). From different liquid biofuels, production of lignocellulosic bioethanol is emerging as a preferred option, because lignocellulosic raw materials represent the most abundant global resource for production of liquid biofuels (Lin and Tanaka, 2006; Talebnia et al., 2010; Kim and Kim, 2014). Also, because demand for biofuels has been increasing together with demand for food, EC made an amendment to the Renewable Energy Directive 2009/28/EC, stating that at least half of the 10% share of renewable fuels in the transportation sector must be produced from non-food based resources (European Commission, 2012).

In order to avoid the conflict between food versus fuel for lignocellulosic bioethanol production from available raw materials in Estonia, attention has to be turned towards utilization of agricultural residues, currently out of use agricultural land and to the utilization of biomass from semi-natural grasslands. The most abundant agricultural residue in Estonia is straw according to Statistics Estonia (2014), which is mostly left on the fields. Only a small percentage is used for animal fodder and bedding or for direct combustion. From different semi-natural grasslands, floodplain or alluvial meadows produce the highest above ground biomass yields with relatively little environmental impact and with no competition from the food market for this biomass resource (Melts, 2014). Also, Estonia has a large share of agricultural land that is currently out of use thus, providing the possibility of

cultivating high biomass yield energy crops without a notable impact on food production land usage.

2. REVIEW OF THE LITERATURE

2.1. Classification of biofuels

Biofuels are classified as primary and secondary biofuels based on their type, origin and production technology, see figure 1. The primary biofuels are natural and unprocessed biomass such as wood (wood chips, pellets) and landfill gas. These are mainly used by direct combustion for heating and production of electricity, for example in CHP plants. The secondary biofuels are produced by processing of biomass, for example, ethanol, biodiesel, methanol, biogas etc. (Nigam and Singh, 2011). The secondary biofuels are divided into first, second and third generation biofuels on the basis of raw material and technology used for their production.
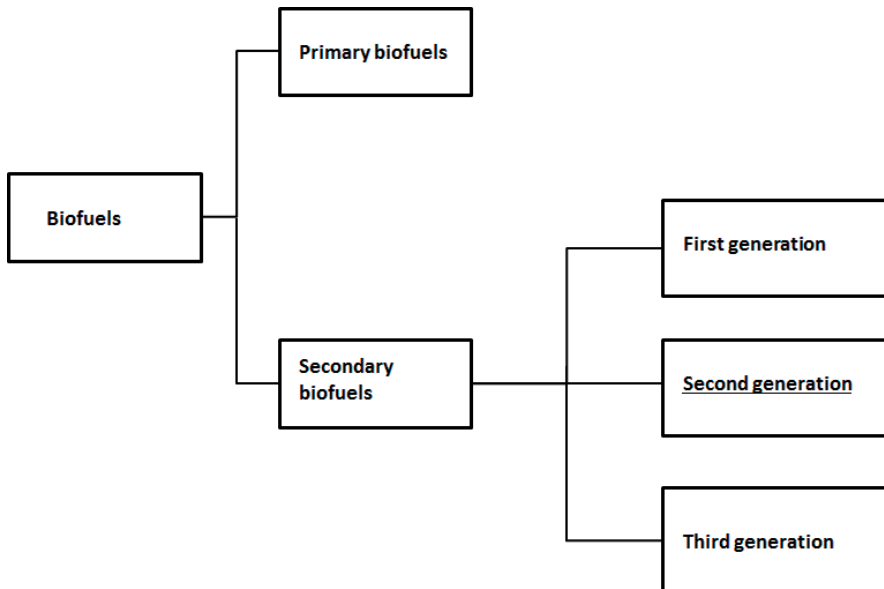

Figure 1. Classification of biofuels.

First generation biofuels are produced by fermentation of starches and sugars (ethanol) or by transesterification of plant oils and animal fats (biodiesel) (Larson, 2008). Problem with the production of first generation biofuels is that they have high GHG emissions and they also compete directly with food market for raw materials and land.

Second generation biofuels are produced from lignocellulosic biomass, such as agricultural residues, grass and wood. It involves biological or thermochemical processing of the material, for example enzymatic hydrolysis, anaerobic digestion and gasification (Nigam and Singh, 2011). The most widely produced second generation biofuels in the world are cellulosic bioethanol, biogas and Fischer-Trops biodiesel (GRFA, 2014). Second generation biofuels have low GHG emissions and they do not compete directly with food production. Negative side of the second generation biofuel production is that it needs sophisticated equipment and energy intensive processing thus, making the product expensive.

Third generation biofuels (bioethanol, biodiesel and hydrogen) are produced from microalgae and other microorganisms. Microalgae are very fast growing organisms, which are able to produce different products like hydrogen, starch or oil. Some microalgae strains contain up to 80% of oil, which can be used to produce biodiesel. Algal biomass also contains cellulose, which can be used for bioethanol production in combination with hydrolysis or gasification (Patil et al. 2008). So far, production of third generation biofuels has been largely restricted to laboratories and demonstration plants. Large scale cultivation in open ponds can be done only in regions with suitable light and climatic conditions, because microalgae need warm and stable environment with plenty of light. In colder regions microalgae are grown in photobioreactors with artificial lighting and heating, because for most of the year there is not enough sunlight and the outside temperature is too low for rapid growth of algal biomass. Need for energy intensive and expensive photobioreactors makes production of third generation biofuels economically unfeasible at the moment. Nevertheless, development of cost effective photobioreactors is continuously ongoing, because microalgae offers much higher fuel production potential than any other energy crops (Chen et al., 2011).

2.2. Composition of lignocellulosic biomass

Lignocellulosic biomass consists of cellulose, hemicellulose, lignin, proteins, sugars and inorganic components, from which cellulose, hemicelluloses and lignin make up approximately 75-85% of dry matter

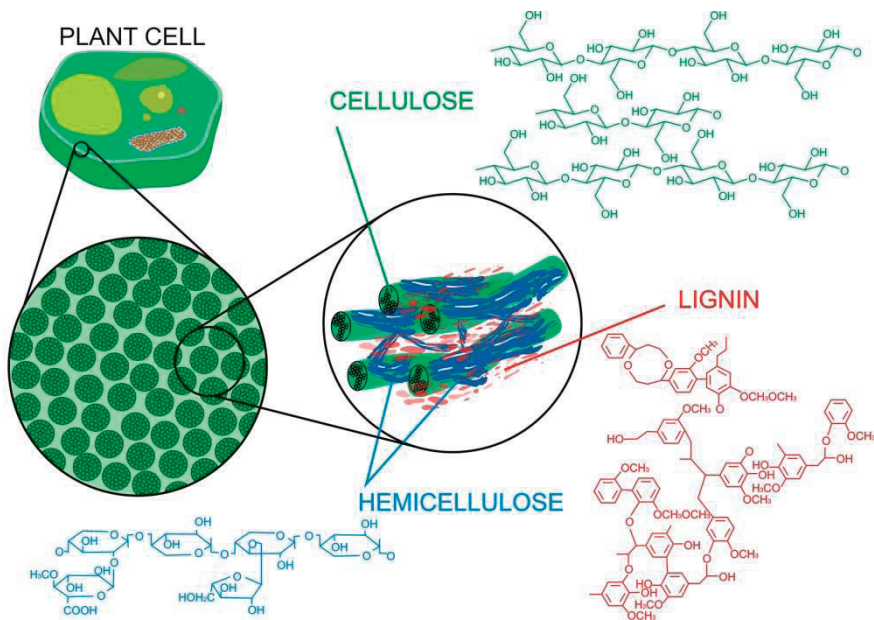(Dey and Harborne, 1997). Structure and biochemical composition of plant cell wall is displayed in figure 2.

Figure 2. Biochemical composition of lignocellulosic biomass (modified from Kowsari, 2013).

Cellulose $(C_6H_{10}O_5)_n$ is a linear polysaccharide which consists of D– glucose monomers. Cellulose does not dissolve in water and is difficult to biodegrade because of its crystalline structure and the beta-linkages between glucose monomers. Cellulose fibres are found mostly in plant cell wall constructions (Guenet, 2008). Lignocellulosic biomass has a cellulose content of 25–55%, depending on plant species.

Hemicellulose $(C_5H_8O_4)_n$ is a branched polysaccharide which consists of different C_6 and C_5 sugar monomers (glucose, mannose, arabinose, xylose etc.)(Laine, 2005). Hemicellulose is mostly found in plant cell walls together with cellulose. Because of its branched and amorphous structure, it is easily degradable by microorganisms or by thermochemical and -physical processes. Hemicellulose content of lignocellulosic biomass is on average 20–40% (Mood, 2013).

Lignin is a complex biopolymer that consists of different aromatic compounds. The main building components of lignin are

17

hydroxycinnamyl alcohols which are synthesized from phenylpropane (Vanholme, 2010). Lignin is an integral part of the secondary cell wall of the plants covering cellulose fibres and hemicellulose. Lignin is relatively hydrophobic polymer and it is one of the most slowly decomposing components of vegetation because of its cross-linked structure. This also minimizes the biodegradability of cellulose and hemicellulose by microbial enzymes. Lignin content of lignocellulosic biomass is on average 10–20% (Sjöström, 1993).

2.3. Biomass conversion technologies

Cellulosic ethanol production is a complex process compared to first generation ethanol production from grain, corn or sugarcane. Conversion process of lignocellulosic biomass to bioethanol is illustrated in figure 3.
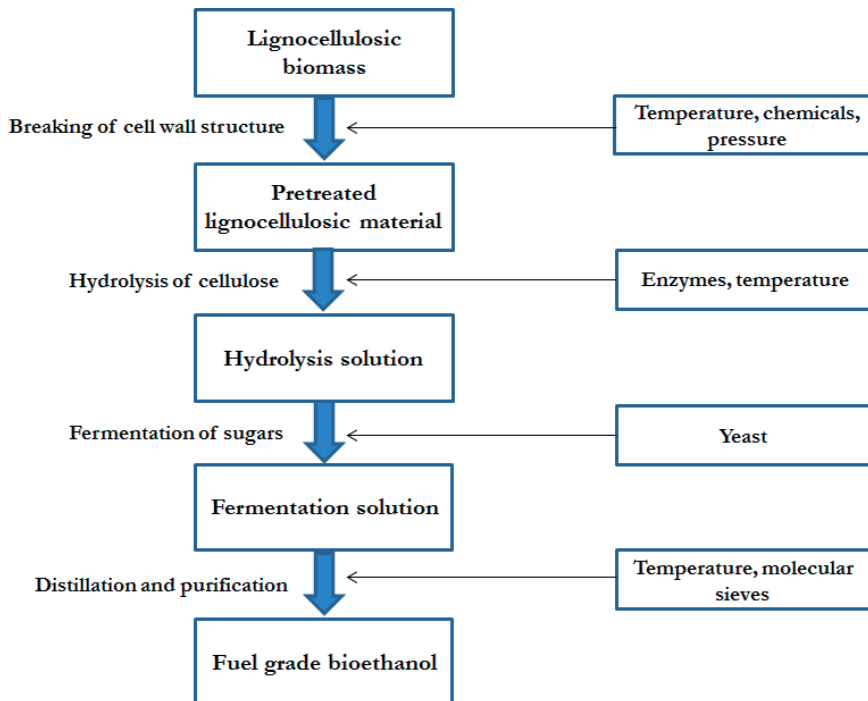

Figure 3. Schematic of the biomass conversion into bioethanol.

Firstly, it is necessary to reduce the particle size of biomass fibres, for example by milling, to achieve better mixing and handling of material. Then, it is necessary to break the cell wall structure including lignin and hemicellulose matrix covering cellulose fibres, and to disrupt the crystalline structure of cellulose. This disruption of plant cell structure is achieved by using hydrothermal or -chemical pretreatment processes, which include applying high temperature, pressure and/or chemicals to the milled biomass (Dvivedi, 2009; Kim, 2011; Christensen and Gerlach, 2010). After the cellulose fibres have been exposed, it is possible to hydrolyse the cellulose to sugar monomers by adding enzymes to the pretreated lignocellulosic material. Hydrolysis step is usually followed by fermentation process by adding yeast to the solution. Finally, the fermented solution is distilled and then purified with molecular sieves to get high quality fuel grade bioethanol.

2.3.1. Pretreatment methods

The most commonly used pretreatment methods for lignocellulosic bioethanol production are dilute acid pretreatment, alkaline pretreatment, steam explosion, carbon dioxide explosion and ammonia fibre explosion pretreatments.

2.3.1.1. Pretreatment with dilute acid

Pretreatment with dilute acid has been one of the most widely used methods for pretreatment of lignocellulosic material in the past. This method uses simple equipment and moderate operating conditions. Dilute acid pretreatment hydrolyses efficiently hemicellulose removing it from plant cell walls, but does little damage to lignin. Lignin remains in plant cell walls and continues to block the access of enzymes to cellulose fibres. Most commonly sulphuric acid is used in dilute acid pretreatment because of its lower price and higher catalytic activity, but the use of nitric, hydrochloric, phosphoric and organic acids have also been reported. In the pretreatment with dilute acid, 0.5 – 2.5% (w/w) acid solution is added to the biomass to hydrolyse hemicellulose during 5 – 60 minutes at 130 – 210°C (Sarkar et al., 2012). Higher temperatures require shorter time of pretreatment and give better conversion yields (Yang, 2009; Dien, 2006). Downside of the dilute

acid pretreatment method is a large consumption of chemicals, slow conversion rate and formation of byproducts that are inhibitory for the following enzymatic hydrolysis and fermentation processes (Behera et al., 2014).

2.3.1.2. Pretreatment with alkaline solution

Pretreatment with alkaline solution uses similar equipment, but lower pretreatment temperatures than dilute acid pretreatment. Alkaline solution removes lignin and part of the hemicellulose from plant cell walls thus, increasing the accessibility of enzymes to cellulose fibres in later phases of hydrolysis. Most of the cellulose and hemicellulose is left in an insoluble polymeric form, but alkaline pretreatment causes chemical swelling of cellulose and hemicellulose thus, making the material more porous, which in addition makes it easier for enzymes to degrade the material in the following hydrolysis process (Dwivedi et al., 2009; Hamelinck, 2005; Mosier, 2005, Mood et al., 2013). Alkaline pretreatment process uses alkali such as NaOH, KOH and $Ca(OH)_2$ and temperatures of 85 – 185ºC (Sarkar et al., 2012; Behera et al., 2014, Ibrahim, 2012). Pretreatment with alkali has been reported to give better ethanol yields than pretreatment with dilute acid due to better fermentation efficiency, because formation of inhibitory by-products like hydroxymethylfurfural (HMF) is avoided (Badiei et al., 2014). Downside of the method is a formation of large amounts of salts that need removal or treatment. Also, chemicals used in alkaline pretreatment are more expensive than the ones used in dilute acid pretreatment (Gupta, 2008; Behera et al., 2014). Pretreatment with alkali is most commonly used for biomass with high lignin content.

2.3.1.3. Steam explosion pretreatment

Steam explosion pretreatment is at the moment, one of the most widely used pretreatment methods for production of bioethanol, because it doesn't consume chemicals and it gives high bioethanol yields up to 85% from the theoretical ethanol yield (Ballesteros et al., 2006). During steam explosion, lignocellulosic biomass is heated at elevated temperatures of 150 – 280°C with high pressure steam. After

a few minutes of incubation time, the heated biomass is subjected to explosive decompression, which physically and chemically changes the lignocellulosic biomass structure and composition (Foody et al., 2000; Alvira et al., 2010; Singh et al., 2015). When biomass is exposed to high temperature steam, hemicellulose is hydrolysed, part of lignin is solubilized and cellulose binding is reduced and structure loosened (Cantarella et al., 2004; Nguyen, 2010). Under explosive decompression, superheated water flashes into steam and steam volume expands generating an impact force which destroys biomass cell structure, tears materials into small pieces, separates cellulose fibre-bundles and thereby, re-distributes lignin and exposes cellulose to enzyme activity (Zhang and Chen, 2012). The main drawbacks of steam explosion method are the formation of HMF and organic acids during pretreatment, which can inhibit the fermentation process, and the difficulty to produce industrial scale equipment that could withstand exposure to high temperatures and rapid pressure changes (Chen and Qiu, 2010; Alvira et al., 2010).

2.3.1.4. CO_2 explosion pretreatment

CO_2 explosion pretreatment is similar method to steam explosion and to other explosive decompression type methods. CO_2 explosion pretreatment uses lower temperatures of 35–165°C, than steam explosion. CO_2 is a non-toxic and non-flammable compound, which makes this method environmentally friendly (Mood et al., 2013; Kim and Hong, 2001). Under high pressure CO_2 molecules can penetrate cell walls and form carbonic acid in water, which hydrolyses hemicellulose (Behera et al., 2014; Zhang and Wu, 2013). Under explosive decompression CO_2 volume expands rapidly destroying the biomass structure, re-distributing lignin and exposing cellulose fibres similarly to steam explosion process. CO_2 explosion pretreatment have been reported to give high glucose yields from wood, up to 85% (Kim and Hong, 2001), however it is less suitable for pretreating herbaceous biomass, for example switchgrass, producing glucose yields of 25 – 40% reported by Narayanaswamy et al., 2011. Another negative aspect of CO_2 explosion is the need for sophisticated equipment that could withstand high CO_2 pressures and rapid pressure changes.
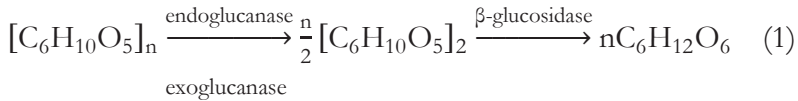
2.3.1.5. Ammonia fibre explosion (AFEX) pretreatment

In AFEX pretreatment liquid ammonia is added to biomass and then the mixture is exposed to temperatures of 70–200°C and pressures of 7–30 bar (Dale et al., 2009; Bals et al., 2010). In its principle, AFEX is similar method to steam explosion and CO_2 explosion methods, because the plant cell wall disruption is caused by the explosive decompression that destroys the cell wall structure. In addition, liquid ammonia removes lignin and reduces the crystallinity of cellulose. AFEX pretreatment is reported to give very high glucose yields of over 90% when used for pretreating grasses, energy crops and agricultural residues (Bals et al., 2010; Teymouri et al., 2005; Murnen et al., 2007; Bradshaw et al., 2007). Disadvantage of AFEX pretreatment is the high ammonia loading, cost of ammonia and environmental concerns that utilization of ammonia produces. Ammonia loading used in AFEX pretreatment is typically 1 kg of ammonia per kg of dry biomass (Brodeur et al., 2011; Alizadeh et al., 2005; Teymouri et al., 2004; Chundawat et al., 2007). Also, AFEX pretreatment is ineffective in pretreatment of lignocellulosic material with high lignin content (Agbor et al., 2011).

2.3.2.   Enzymatic hydrolysis

Different physico-chemical pretreatment processes are usually followed by enzymatic hydrolysis to depolymerise lignocellulosic biomass and to convert it to fermentable sugars (Modenbach & Nokes, 2013). Enzymatic hydrolysis has low capital and operational costs, because this process does not need sophisticated equipment or high temperatures (Mood et al., 2013). Enzymatic hydrolysis can be carried out with a total solid loading of up to 20% (w/w), but if the solid loading is too high then equal distribution of enzymes in the hydrolysis mixture becomes difficult to achieve, resulting in lower sugar conversion rate. Hydrolysis is carried out at temperatures of 20–75°C by different cellulase enzymes which are usually produced by lignocellulose degrading bacteria or fungi, for example strains of *Trichoderma* or *Aspergillus* (Rosendahl, 2013; Maitan-Alfenas et al., 2015). As a first step, cellulose fibres are degraded by different enzymes to smaller units like cellobiose, which is then further degraded into glucose monomers (Sun and Cheng, 2002). This process is

accomplished by three different types of enzymes as seen in equation 1. Endoglucanases break down interactions in the crystalline structure of cellulose. Exoglucanases hydrolyse cellulose fibres to simple sugars like cellobiose. β-glucosidases hydrolyse disaccharides like cellobiose into glucose monomers (Harun & Danquah, 2011; Li et al., 2009; Yeh et al., 2010).

$$[C_6H_{10}O_5]_n \xrightarrow{\text{endoglucanase}} \frac{n}{2}[C_6H_{10}O_5]_2 \xrightarrow{\text{β-glucosidase}} nC_6H_{12}O_6 \quad (1)$$

$$\text{exoglucanase}$$

The main factors that affect the hydrolysis rate of cellulose are accessibility of cellulose fibres to enzymes, enzyme activity and presence of inhibiting compounds. The speed of enzyme reactions are described by figure 4 and by Michaelis-Menten equation (equation 2) (Horton et al., 2002; Harun and Danquah, 2011):

$$V = \frac{V_{max}[S]}{K_m + [S]} \qquad (\text{mol s}^{-1}\text{ L}^{-1}) \ (2)$$

where V represents the speed of reaction, V_{max} shows the maximum speed of reaction, S represents the substrate concentration and K_m is a kinetic constant that equals to substrate concentration, at which half of the maximum speed $\left(\frac{V_{max}}{2}\right)$ of reaction is achieved.
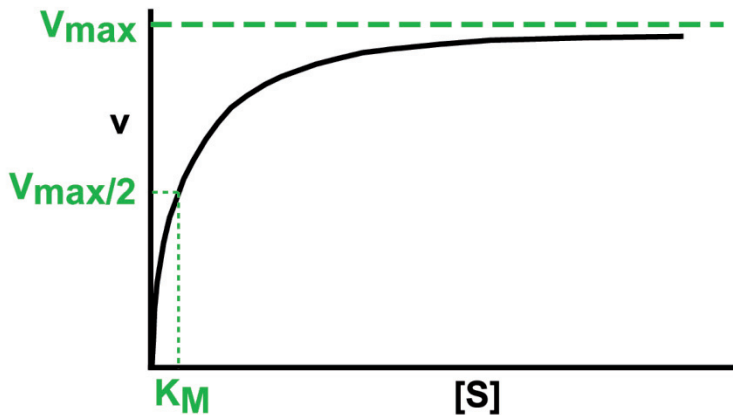

Figure 4. Correlation between enzyme reaction speed and substrate concentration (Horton et al., 2002).

When we take into account the negative effect of inhibition to the speed of enzyme reactions, we need to modify the Michaelis-Menten equation and include the product concentration P_c and inhibition constant k_i as seen in equation 3.

$$V = \frac{V_{max}[S]}{K_m(1 + [P_c]/k_i) + [S]} \qquad \text{(mol s}^{-1}\text{ L}^{-1}\text{)} \quad (3)$$

2.3.3. Fermentation

C6 sugars like glucose, mannose and galactose are easily fermented to alcohols with high efficiency, by a number of naturally occurring microorganisms. Fermentation of glucose to ethanol and carbon dioxide is described by equation 4.

$$C_6H_{12}O_6 \xrightarrow{\text{yeast}} 2C_2H_5OH + 2CO_2 \qquad (4)$$

One of the most commonly used microorganisms for fermentation is yeast *Saccharomyces cerevisiae*. Other microorganisms that are capable of fermenting C6 sugars into alcohol are for example *Zymomonas mobilis, Pachysolen tannophilus, Pichia striptis* and *Escherichia coli* (Andersen et al., 2011; Maitan-Alfenas et al., 2015). Fermentation of C5 sugars is much more difficult process with slow fermentation rates and low alcohol yields and only a few naturally occurring microorganisms are capable of this. One yeast strain that is used for xylose fermentation is *Pachysolen tannophilu*. Fermentation is often carried out in separate steps, because microorganisms that can ferment C5 sugars are usually very sensitive to inhibitors and rising alcohol concentration (Dwivedi et al., 2009; Shleser, 1994).

Fermentation is carried out at temperatures of 20 – 40°C and for a period of 48-168 hours although, over 90% of the C6 sugars are usually fermented during the first 48 hours (Thangavelu et al., 2014). Fermentation of sugars for bioethanol production is followed by distillation and purification steps to achieve fuel grade quality ethanol.

2.4. Overview of energy crops used for bioethanol production

The selection of energy crops was done based on the plant species grown on the experimental fields of Estonian University of Life Sciences with the inclusion of agricultural by-products and biomass from semi-natural grasslands like straw and floodplain meadow hay, which do not compete with food production.

Sunflower (*Helianthus annuus L.*) is a perennial plant native to Northern and Southern America. Sunflowers are mostly grown for their seeds, which are used for food and production of sunflower oil. Above ground biomass of sunflowers has little use, but since sunflower is relatively simple to cultivate, is fast growing and supresses weeds, it has a potential for the use as a biomass crop. Sunflowers' stems grow on average 3 – 4 metres tall and have a mean above ground biomass production of 7.7 – 13.5 t ha^{-1} in Estonian climatic conditions (Lauk et al., 2009).

Amur silver grass (*Miscanthus sacchariflorus L.*) is a perennial grass native to Eastern Asia, but it also grows well in Estonian climatic conditions. Perennial grasses from the *Miscanthus* family are reported to have a biomass yield of up to 25 t ha^{-1} in warmer regions (Voigt et al., 2012). Silver grass has been reported to give ethanol yields of 60.8 – 127.3 g kg^{-1} depending on the pretreatment and production conditions (Vogel et al., 2011).

Jerusalem artichoke (*Helianthus tuberosus L.*) is a perennial plant native to Northern America growing up to 3 m tall. It is mainly cultivated for its roots, which are used for food. Jerusalem artichokes produce up to 28 tons per hectare of green biomass (9.5 t ha^{-1} of DM), but the stems contain little nutrients and are not used as animal feed (Purdue University, 2014).

Energy grass cultivar Szarvasi-1 has been developed for energetical purposes by Agricultural Research and Development Institute P.U.C. in Hungary (ARADI, 2014). It is mostly used for briquetting and direct burning, but it can also be used for biogas and bioethanol production (Sipos, 2014).

Industrial hemp (*Cannabis sativa L.*) is a herbaceous annual plant that develops a rigid woody stem, can grow nearly 5 metres tall and produces 6.1 – 21.5 t ha^{-1} of above ground biomass on dry matter basis (Ehrensing, 1998). Hemp has high cellulose content of 35 – 55%, which makes it a suitable crop for bioethanol production. Industrial hemp has been reported to give bioethanol yields of up to 155 g kg^{-1} of biomass when using a combined two stage steam/dilute acid pretreatment method (Kuglarz et al., 2014). Hemp fibres have many other industrial applications too, for example it is used in composite materials, in textile or pulp and paper production (Gonzales-Garcia et al. 2012). Hemp has also been reported as a good solid fuel for combustion.

Wheat is most widely grown grain in Europe. For example in Estonia, grain was cultivated on 311 100 ha in 2013, from which 124 200 ha was wheat (Statistics Estonia, 2014). Annual wheat straw production in Estonia is approximately 385 000 tons, from which about half could be available for bioethanol production, because wheat straw has high cellulose content of up to 47% and has been reported to give ethanol yields of approximately 124 g kg^{-1} of biomass (Eisenhuber et al., 2013). The rest of wheat straw is used for animal bedding or should be left on the fields to improve soil conditions.

Floodplain meadows are semi-natural grasslands with high biodiversity that need regular maintenance (Sammul et al., 2000; Kukk and Sammul, 2006). There is nearly 20 000 hectares of floodplain meadows in Estonia, which annually produce on average 5.7 t ha^{-1} of biomass (Heinsoo et al., 2010). This biomass has had little use and so provides an available resource for bioethanol production.

3. AIMS OF THE STUDY

The aim of this thesis was to analyse the bioethanol production potential of different energy crops grown in Estonian climatic conditions and to find the optimum pretreatment method and conditions for bioethanol production from selected energy crops in order to help to fulfil the target of reaching 10% share of biofuels in the Estonian transportation sector.

The main objectives in the present thesis are:

1) To analyse the above ground biomass yield and biochemical composition of different energy crops and their corresponding bioethanol yields (Papers **I-V**).

2) To study how harvesting time can influence the bioethanol yield of energy crops (Paper **I, III**).

3) To find the optimum pretreatment method and conditions for bioethanol production from selected energy crops (Paper **II, IV, V**).

4) To modify or develop a pretreatment method that would be cost-effective and would provide high bioethanol production efficiency at moderate pretreatment conditions (Paper **V**).

5) To calculate the potential annual quantity of bioethanol production in Estonia based on experimental conversion efficiencies and above ground biomass yields (Paper **I, II, V**).

4. MATERIALS AND METHODS

Different biomass types and pretreatment methods were analysed in this work. As a first step, biomass was milled to reduce the particle size and to improve the handling and mixing of biomass. Secondly, biomass was hydrothermally pretreated by dilute acid, alkaline, steam explosion or N_2 explosion pretreatment methods in order to break the plant cell wall structure and expose cellulose fibres. As a third step, pretreated biomass was hydrolysed to C6 sugars by adding enzyme mixture Accellerase 1500. Finally, dry yeast Saccharomyces cerevisiae was added to hydrolysis solution and glucose in hydrolysis solution was fermented to ethanol. All experiments were done at least in triplicates unless indicated differently. Only steam explosion experiments were done in duplicates.

4.1. Biomass

To investigate the growth of different energy crops in Estonian climatic conditions and their suitability for bioethanol production, a collection of experimental fields was established in 2010 at the Institute of Agricultural and Environmental Sciences of Estonian University of Life Sciences on Haplic Luvisol (Hypereutric) soil near Tartu, Estonia. The plot size for each plant species was 5 m^2 without replications (Paper **I**). The plant species grown in the experimental fields were: industrial fibre hemp (*Cannabis sativa L.*) cultivar USO-31, sunflower (*Helianthus annuus L.*) cultivar Wielkopolski, Jerusalem artichoke (*Helianthus tuberosus L.*), Amur silver-grass (*Miscanthus sacchariflorus L.*), and energy grass (Szarvasi-1). During the vegetation period no pesticides were applied. The height of the plants was determined before the harvest (Paper **I**). Samples for chemical analysis and bioethanol experiments were taken in autumn 2010 - 2012, when the vegetation period had already finished and plants had reached maturity.

In addition, industrial hemp samples were also harvested from July 28th to September 8th, 2011, and samples of Jerusalem artichoke were taken on September 10th, 2010 from the experimental fields of Estonian University of Life Sciences (Paper **III**). Hemicellulose, cellulose and lignin contents of aboveground biomass samples were determined in the Laboratory of Plant Biochemistry of Estonian University of Life Sciences. Wheat straw samples were harvested in August, 2011, from

fields in Vara, Tartumaa (Paper **IV**). Floodplain meadow hay samples were harvested in July, 2012, from the floodplains of Emajõgi, Tartumaa (Paper **II**).

Harvested biomass was dried and stored in dry conditions before milling (moisture content of samples was $10 \pm 1\%$). All samples were milled to a particle size of $1 - 3$ mm with Cutting Mill SM 100 comfort (Retsch GmbH) and Cutting Mill ZM 200 (Retsch GmbH). Milled samples were stored at a room temperature $22 \pm 3°C$. Dry matter content of samples was $90 \pm 1\%$ (Papers **I-V**).

4.2. Pretreatment

4.2.1. Dilute acid pretreatment

Sample size was 75 g of pre-dried and milled biomass due to small quantity of sample material to which 750 mL of 1% H_2SO_4 (w/w) solution was added. Mixture was inserted into a 2000 mL reactor vessel, where all samples were heated for $t = 60$ minutes at a temperature $T = 130 - 150 \pm 3°C$ and a pressure of $P = 3 - 5$ bar. Pressure rise occurs because of the temperature rise in a hermetically sealed reactor vessel, no additional pressure is added. Samples were cooled below $50°C$ and pH of the mixture was neutralized using $Ca(OH)_2$ before the enzymatic hydrolysis step, because enzymes used in the hydrolysis are inactivated when temperature $T > 70°C$ or when pH < 4 or pH > 7 (Paper **I**).

For wheat straw samples different approach was used. Dilute sulphuric acid, hydrochloric acid or nitric acid solution was used for pretreatment. The sample size was 100 g of pre-dried and milled wheat straw to which 1000 mL of 1% acid (w/w) solution was added. Mixture was inserted into a 2000 mL reactor vessel, where all samples were heated for $t = 60$ minutes at a temperature $T = 130 - 150 \pm 3°C$ and a pressure of $P = 3 - 5$ bar (Paper **IV**). Samples were cooled to a temperature below $50°C$ and K_2CO_3 was added to neutralize the pH. K_2CO_3 was used in later experiments instead of $Ca(OH)_2$, because K_2CO_3 is cheaper and reduces the cost of bioethanol production.

4.2.2. Alkaline pretreatment

Dilute potassium hydroxide solution was used for alkaline pretreatment. The sample size was 100 g of pre-dried and milled wheat straw to which 1000 mL of 1% alkaline (w/w) solution was added. All samples were heated for $t = 60$ minutes at a temperature $T = 130 - 150 \pm 3°C$ and a pressure of $P = 3 - 5$ bar. Samples were cooled to a temperature below 50 °C and HCl was added to neutralize the pH (Paper **IV**).

4.2.3. Steam explosion pretreatment

Steam explosion was used for the pretreatment of floodplain meadow hay and wheat straw samples. Sample size was 900 g of pre-dried and milled biomass, which was soaked in 900 g of distilled water (Paper **II**). Pretreatment was performed in a laboratory scale steam explosion system (see figure. 5) at the University of Applied Sciences Upper–Austria. Steam explosion was carried out at temperatures $T = 150 - 200 \pm 1°C$ and incubation times of $10 - 30$ minutes. Pretreated material was then dried at temperature $T = 40 \pm 1°C$ to a dry matter content of $95 \pm 1\%$ (Paper **II**).
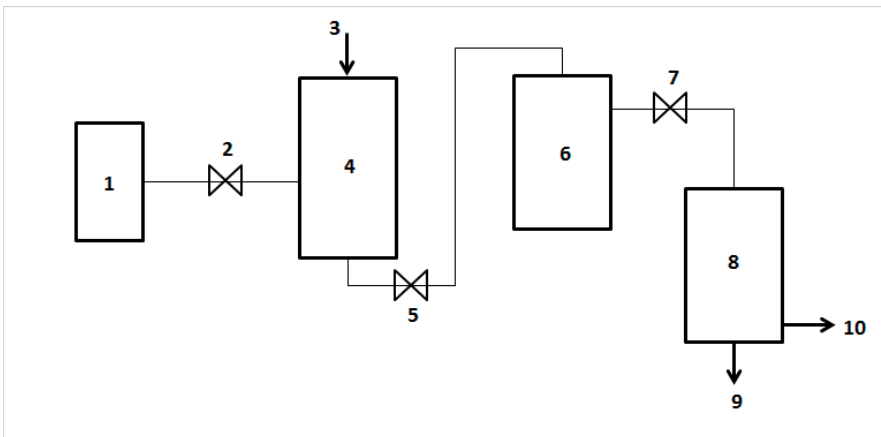

Figure 5. Schematic of the steam explosion system (1- steam generator, 2- steam control valve, 3- biomass input, 4- reactor vessel, 5- pressure release valve, 6- steam expansion vessel, 7- pressure valve, 8- collection vessel, 9- material extraction, 10- condensate drain).

4.2.4. N$_2$ explosion pretreatment

In the N$_2$ explosion pretreatment sample of 100 ± 0.1 g of pre-dried and milled hay was soaked in 500 ± 3 g of distilled water and inserted into the reactor vessel. Reactor vessel was pressurized with N$_2$ gas to a pressure of $P = 30$ bar. Samples were heated in the reactor for $t = 15 - 120$ minutes to temperatures of $T = 70 - 210 \pm 3°C$ with a total incubation time of 180 ± 5 minutes (Paper **V**). Total incubation time includes the time that is needed for reaching the desired temperature in the pressurised reactor and for cooling it down before the explosive decompression. After reaching the desired temperature, reactor was cooled down to $< 90 °C$ and pressure was released through the release valve (see figure 6). Patent application P201400050 of nitrogen explosion pretreatment method for disruption of cellular structure of biomass has been submitted to the Estonian Patent Office.
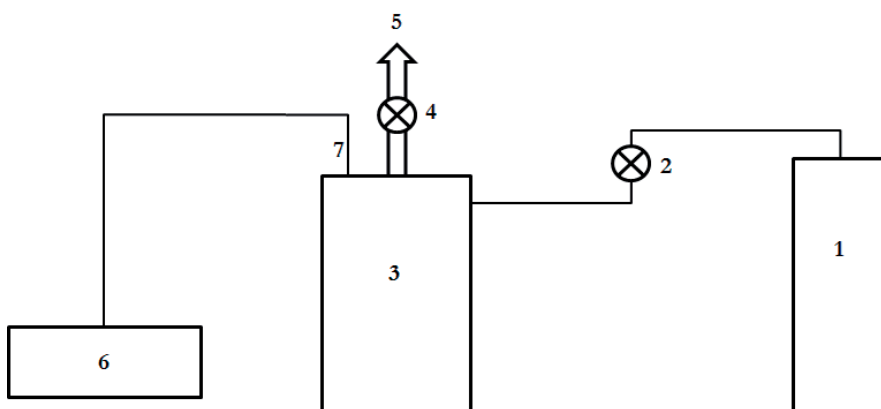
Figure 6. Schematic of the nitrogen explosion pretreatment system (1- nitrogen tank, 2- pressure control valve, 3- reactor vessel with electric heating, 4- pressure release valve, 5- ventilation system, 6- controller unit, 7- thermocouple).

4.3. Hydrolysis

Pretreatment phase was followed by enzymatic hydrolysis with enzyme complex Accellerase 1500. Enzyme mixture was added to the sample at a ratio of 0.3 mL per g of biomass. Hydrolysis of the pretreated material was carried out in 1000 mL flasks. The samples were

incubated for 24 h at 50°C in a shaking incubator at rotational speed of 250 rpm (Papers **I-V**).

4.4. Fermentation

Fermentation of glucose into ethanol was performed using dry yeast *Saccharomyces cerevisiae* in 1000 mL glass bottles that were sealed with fermentation tubes. 2.5 g of yeast per 100 g of biomass was added to the solution and fermentation was carried out at room temperature for duration of 168 h (Papers **I, III-V**).

For samples pretreated with steam explosion in University of Applied Sciences Upper–Austria, a slightly different method for fermentation was used. Volume of fermentation medium was 500 mL. Fermentation medium had a pH = 4.6 which was adjusted with H_2SO_4. Fermentation medium contained 100 mL hydrolysate, 2 mL $CaCl_2 \cdot 2H_2O$, 2 mL KH_2PO_4, 2 mL $MgSO_4 \cdot 7H_2O$, 0.44 g $(NH_4)_2HPO_4$. Yeast suspension (2 mL) was added to the solution and fermentation was carried out at 30°C for 120 hours (Paper **II**).

4.5. Analysis

Dry matter content of samples was analysed with a moisture analyser Ohaus MB 45. The fibre analysis (cellulose, hemicellulose and lignin) was performed according to the methods of Association of Official Analytical Chemists (AOAC 973.18) and methods by Tecator company (fibre determination using Tecator, Part No. 1000 1217, Serial No. 1706, U=200-240 V, f=50/60 Hz, P_e=1000 W). The results of fibre analysis were also checked with acid hydrolysis. Acid hydrolysis was done according to the methods of National Renewable Energy Laboratory of USA for determination of structural carbohydrates and lignin in biomass (NREL, 2012) (Papers **I-V**).

Glucose and ethanol concentration in sample solutions were measured by Analox GL–6 analyser (Analox Instruments) (Papers **III-V**).

Saccharides, organic acids, furans and ethanol concentrations in sample solutions analysed in University of Applied Sciences Upper–Austria,

were measured by high performance liquid chromatography (HPLC). The HPLC system Agilent Technologies 1200 Series with a Varian Metacarb 87 H column (300·7.8 mm) at 65°C, H_2SO_4 (c = 5 mmol L^{-1}) eluent and an isocratic flow rate of 0.8 mL min^{-1} was used. The signals were acquired with a refractive index (RI) and a UV–detector at 210 nm wavelength (Eisenhuber et al., 2013) (Paper **II**).

Pictures of biomass particle surfaces were taken by scanning electron microscopes Zeiss EVO LS15 (Carl Zeiss AG) and Tescan VEGA (Tescan).

Theoretical quantity of glucose was calculated according to equation 5 and on the assumption, that 100% of cellulose is converted to glucose monomers, where m_{gluc} – theoretical quantity of glucose in grams; C_{cel} - cellulose content in percentages; m_{sample} – quantity of sample in grams, C_{DM} – dry matter content in percentages; 1.11 – cellulose to glucose conversion factor based on stoichiometric biochemistry of hydrolysis (Lu et al., 2012).

$$m_{gluc} = \frac{m_{cel}}{100} \times 1.11 \times m_{sample} \times \frac{C_{DM}}{100} \qquad \text{(g) (5)}$$

Theoretical bioethanol potential was calculated according to equation 6 on the assumption, that 100% of glucose is converted to ethanol without the formation of by-products, where B_{tbp} – is theoretical bioethanol potential in grams of pure ethanol per kilogram of biomass dry matter; m_{gluc} – is theoretical quantity of glucose in grams; 0.51 – the conversion factor of glucose to ethanol based on stoichiometric biochemistry of fermentation seen in equation 4.

$$B_{tbp} = 0.51 m_{gluc} \qquad \text{(g kg}^{-1}\text{) (6)}$$

Theoretical bioethanol potential per area was calculated according to equation 7, where B_{by} – experimentally measured biomass yield in grams per m^2; B_{tbpa} – theoretical bioethanol potential in grams per m^2 of biomass growth area.

$$B_{tbpa} = \frac{B_{by}}{1000} \times B_{tbp} \qquad \text{(g m}^{-2}\text{) (7)}$$

Experimental bioethanol yield per area was calculated according to equation 8, where Y_{eby} – experimentally measured bioethanol yield in grams per kg of biomass; B_{by} – experimentally measured biomass yield in grams per m^2; Y_{ebya} – experimentally measured bioethanol yield in grams per m^2 of biomass growth area.

$$Y_{ebya} = \frac{B_{by}}{1000} \times Y_{eby} \qquad \text{(g m}^{-2}\text{)} \quad (8)$$

Hydrolysis efficiency (E_{he}) is calculated according to equation 9, where $m_{E.gluc}$ – experimentally measured quantity of glucose in grams; m_{gluc} – theoretical quantity of glucose in grams calculated from cellulose content; E_{he} – hydrolysis efficiency in percentages.

$$E_{he} = \frac{m_{E.gluc}}{m_{gluc}} \times 100 \qquad (\%) \quad (9)$$

Fermentation efficiency is calculated according to equations 10 and 11, where E_{fe} – fermentation efficiency in percentages; $m_{E.eth}$ – experimentally measured quantity of ethanol in grams; m_{eth} – theoretical quantity of ethanol in grams calculated from experimentally measured quantity of glucose; 0.51 – the conversion factor of glucose to ethanol based on stoichiometric biochemistry of fermentation (Lu et al., 2012).

$$m_{eth} = 0.51 m_{E.gluc} \qquad \text{(g)} \quad (10)$$

$$E_{fe} = \frac{m_{E.eth}}{m_{eth}} \times 100 \qquad (\%) \quad (11)$$

Overall efficiency is calculated according to equation 12, where E_{oe} – overall bioethanol production efficiency in percentages; $m_{E.eth}$ – experimentally measured quantity of ethanol in grams; B_{tbp} – theoretical bioethanol potential in grams per kilogram of biomass dry matter.

$$E_{oe} = \frac{m_{E.eth}}{B_{tbp}} \times 100 \qquad (\%) \quad (12)$$

Averaged results of parallel measurements are used in all figures and corresponding standard deviations are shown by vertical lines in figures or by ± 1 standard deviation in tables. Standard deviations were calculated according to equation 13, where σ – standard deviation; x –

each value of the data set; $\bar{x}$ - statistical mean of the data set; n – number of analysed samples in data set ($2 \leq n \leq 6$).

$$\sigma = \sqrt{\frac{\sum (x-\bar{x})^2}{n}}$$
(13)

Data was processed with programs Microsoft Excel, GraphPad Prism 5 and ezANOVA. Significance is presented with $p < 0.05$ if not indicated otherwise.

5. RESULTS

5.1. Bioethanol production potential of different crops

Above ground biomass growth, together with biochemical composition of different energy crops was measured to assess their potential for biofuels production in Estonian climatic conditions (Paper **I, II, III, IV**). Bioethanol production potential of different crops was calculated from cellulose contents and above ground biomass yields (see figure 7 and table 1). For analysis of experimental bioethanol yields, dilute acid pretreatment was used, except for wheat straw and floodplain meadow hay, where instead steam explosion pretreatment was used. Biochemical composition of analysed energy crops can be seen in table 2.
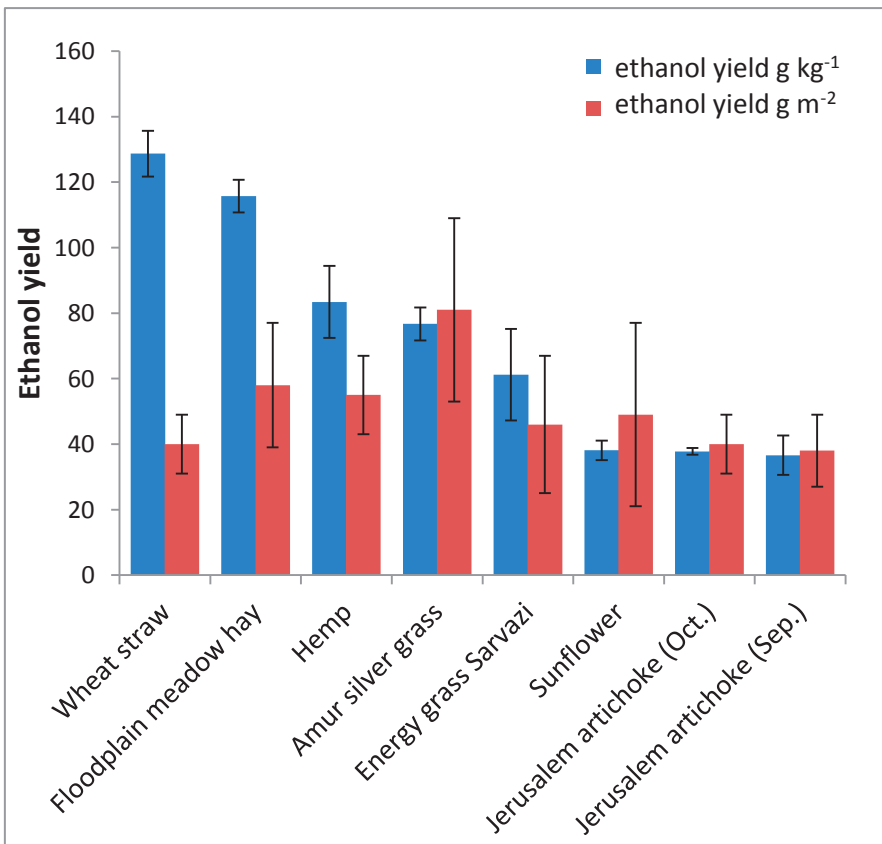

Figure 7. Bioethanol yields of different energy crops in Estonian climatic conditions.

Table 1. Above ground biomass and ethanol yields of different energy crops in Estonian climatic conditions (BY- biomass yield; TBP- theoretical bioethanol potential; TBPA- theoretical bioethanol potential per area; EBY- experimental bioethanol yield; EBYA- experimental bioethanol yield per area).

Sample	BY $g\ m^{-2}$	TBP $g\ kg^{-1}$	TBPA $g\ m^{-2}$	EBY $g\ kg^{-1}$	EBYA $g\ m^{-2}$
Amur silver grass	1050 ± 350	214.2 ± 2.1	225 ± 75	76.7 ± 5.1	81 ± 28
Hemp (Oct)	660 ± 70	274.7 ± 5.7	181 ± 19	83.4 ± 11.3	55 ± 12
Sunflower	1280 ± 730	173.7 ± 1.6	222 ± 127	38.1 ± 3.1	49 ± 28
Energy grass Szarvasi-1	750 ± 250	193.0 ± 4.4	145 ± 48	61.2 ± 14.3	46 ± 21
Jerusalem artichoke (Sept)	1050 ± 250	106.9 ± 1.2	112 ± 27	36.6 ± 5.8	38 ± 11
Jerusalem artichoke (Oct)	1050 ± 250	132.6 ± 1.2	139 ± 33	37.8 ± 0.9	40 ± 9
Wheat straw**	310 ± 50	237.0 ± 1.8	74 ± 12	128.7 ± 8.5	40 ± 9
Floodplain meadow hay (July)**	570 ± 150*	139.0 ± 3.5	79 ± 21	115.7 ± 2.0	58 ± 19

*Floodplain meadow hay biomass yield is average of the results reported in literature (Heinsoo et al., 2010).

**Wheat straw and floodplain meadow hay biomass was pretreated with steam explosion method.

Table 2. Biochemical composition (% in dry mass) of different biomass samples (HCEL- hemicellulose; CEL- cellulose; LIG- lignin).

Sample	Ash	HCEL	CEL	LIG
Amur silver grass	5.37 ± 0.08	30.15 ± 0.55	42.00 ± 0.42	7.00 ± 0.17
Energy grass Szarvasi-1	7.01 ± 0.11	27.33 ± 1.01	37.85 ± 0.86	9.65 ± 0.31
Hemp (Oct.)	5.25 ± 0.08	10.60 ± 0.44	53.86 ± 1.11	8.76 ± 0.19
Sunflower	9.78 ± 0.15	5.18 ± 0.29	34.06 ± 0.31	7.72 ± 0.22
Jerusalem artichoke (Sep.)	5.15 ± 0.13	5.48 ± 0.11	20.95 ± 0.23	5.05 ± 0.11
Jerusalem artichoke (Oct.)	4.56 ± 0.11	4.50 ± 0.09	25.99 ± 0.21	5.70 ± 0.13
Wheat straw	3.57 ± 0.07	31.01 ± 0.38	46.47 ± 0.36	7.94 ± 0.17
Floodplain meadow hay	-	29.15 ± 0.48	27.19 ± 0.69	24.16 ± 0.29

Table 3. Biochemical composition and hydrolysis efficiency (%) of hemp samples harvested at different times (Paper **III**).

Harvesting time	HCEL	CEL	LIG	HE
28th July	10.54 ± 0.24	33.06 ± 0.36	6.48 ± 0.11	53.44 ± 1.43
4th August	14.34 ± 0.42	32.98 ± 0.54	6.29 ± 0.19	59.07 ± 2.11
11th August	10.90 ± 0.33	36.76 ± 0.28	7.37 ± 0.15	51.28 ± 1.66
18th August	9.36 ± 0.21	39.31 ± 0.37	7.60 ± 0.13	51.91 ± 2.21
25th August	9.20 ± 0.29	36.34 ± 0.63	7.98 ± 0.22	46.88 ± 0.97
1st September	10.04 ± 0.36	37.84 ± 0.56	8.16 ± 0.16	50.80 ± 1.27
8th September	8.81 ± 0.19	43.30 ± 0.75	8.15 ± 0.09	46.36 ± 2.37
12th October	10.60 ± 0.44	53.86 ± 1.11	8.76 ± 0.19	51.00 ± 2.84

Statistical ANOVA analysis revealed that there is significant difference ($p<0.05$) in biomass yields of different energy crops when compared to each other except for sunflower where there was no significant difference. Also there was no significant difference ($p>0.05$) in biomass yields between Jerusalem artichokes September and October harvests. Experimental bioethanol yields in grams of absolute alcohol per kg of biomass dry matter are statistically significantly different ($p<0.05$) when compared to each other except for results of sunflower and Jerusalem artichoke September and October harvests where no significant difference was found. There was also no significant difference between experimental bioethanol yields of hemp, Amur silver grass and energy grass Szarvasi ($p>0.05$), but experimental bioethanol yields of these energy crops were significantly different to all other energy crops that were analysed. Statistical ANOVA analysis revealed that there was no significant difference ($p>0.05$) in experimental bioethanol yields per area for different energy crops.

Sunflower had the highest above ground biomass growth rate of 1280 g m^2 of dry matter with a cellulose content of 34.06% (Paper **I**), see table 2. This gives sunflower one of the highest bioethanol potentials per area of 222 g m^{-2}, but since there was no significant difference in biomass yields when compared to other energy crops, then average biomass yields of sunflower can't be confirmed.

Jerusalem artichokes had an above ground biomass production of 1050 g m^2 of dry matter, similar to sunflowers (Paper **I**). Plants had reached full height for harvesting period thus, no significant difference in biomass quantity was measured between Jerusalem artichoke September and October harvests. Jerusalem artichokes September harvest had the lowest cellulose content of 20.95% and lignin content of 5.05% from all analysed plant species.

Amur silver grass had an above ground biomass production of 1050 g m^{-2}, which is nearly 2.5 times lower than in its natural habitat in Eastern Asia, where it has an average biomass production of 2500 g m^{-2} (Voigt et al. 2012). Amur silver grass had a cellulose content of 42.00%, hemicellulose content of 30.15% and lignin content of 7.00% (Paper **I**). Over the average cellulose content together with high above ground biomass yield results in the highest theoretical bioethanol potential of 225 g m^{-2} together with the highest experimental

bioethanol yield of 85 g m^{-2} when using dilute acid pretreatment method.

Energy grass cultivar Szarvasi-1 had an above ground biomass yield of 750 g ha, which did not have significant difference to biomass yields of sunflower or Amur silver grass, but was considerably higher than the above ground biomass yield of wheat straw. Energy grass cultivar Szarvasi-1 had cellulose content of 37.85%, hemicellulose content of 27.33% and lignin content of 9.65% (Paper **I**).

Industrial hemp samples were harvested from the end of July to mid-October and corresponding biochemical composition, together with hydrolysis efficiencies, can be seen in table 3 and figure 8. The latest harvest of industrial hemp gave the above ground biomass yield of 660 g m^{-2} (Paper **I**). Biochemical composition of a plant varies in time, as seen from the analysis results in table 3. Hemp samples harvested in the beginning of August had the lowest cellulose and lignin contents of 32.98% and 6.29%, respectively (Paper **III**). Samples harvested in October had the highest cellulose content of 53.86% and lignin content of 8.76%. Statistical analysis of hemp samples confirmed a significant difference ($p < 0.05$) in cellulose and lignin content of samples harvested at different growth phases. Very high cellulose content gives hemp the highest potential for bioethanol production of 274.7 g per kg of biomass dry matter.
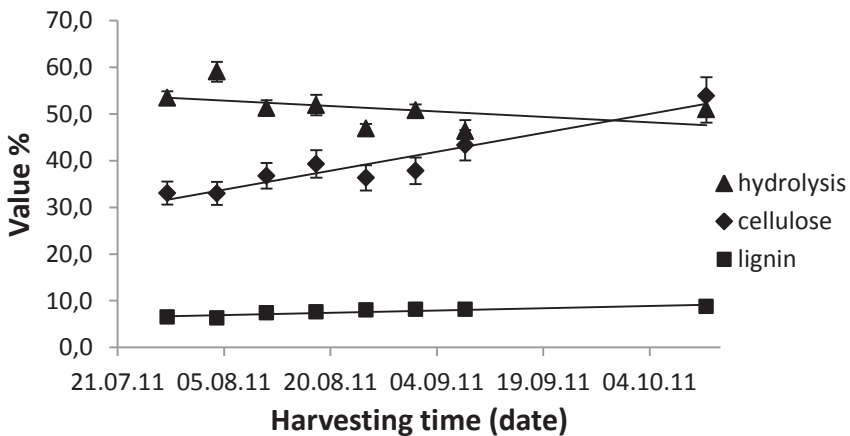

Figure 8. Correlation of cellulose/lignin contents and hydrolysis efficiencies of hemp samples with harvesting times (Paper **III**).

Wheat produced on average of 310 g m^{-2} of straw, which is approximately four times lower than the average above ground biomass yield of sunflower. Since wheat straw has high cellulose content of 46.47%, it gives one of the highest theoretical bioethanol potential of 237.0 g kg^{-1} and experimental bioethanol yield of 128.7 g kg^{-1} (Paper **I, II, IV**).

Floodplain meadows produced on average of 570 g m^{2} of above ground biomass and have the highest lignin content of 24.16% and a cellulose content of 27.19% (Paper **II**). This gives floodplain meadow hay one of the lowest theoretical bioethanol potentials of 139.0 g kg^{-1}.

Experimental results differ by a significant margin from theoretical bioethanol potentials. For example Jerusalem artichoke October harvest and floodplain meadow hay have similar theoretical bioethanol potentials of 132.6 and 139.0 g kg^{-1}, respectively, but the difference in experimental bioethanol yields is approximately 3 times with floodplain meadow hay having a bioethanol yield of 115.7 g kg^{-1} and Jerusalem artichoke (Oct) having a bioethanol yield of only 37.8 g kg^{-1}.

5.2. Comparison of different pretreatment methods

Different pretreatment methods compared to each other in Paper **IV** and **V**, were dilute acid pretreatment with sulphuric, nitric and hydrochloric acids, dilute alkali pretreatment with potassium hydroxide and steam explosion pretreatment method. The novel method for biomass pretreatment developed during this work was nitrogen explosion pretreatment. Raw material chosen for analysis of conventional pretreatment methods was wheat straw and samples collected from the same location and time were used for experiments. Raw material chosen for comparison experiments of steam explosion and nitrogen explosion pretreatment methods was floodplain meadow hay.

5.2.1. Conventional pretreatment methods

From dilute acid pretreatment with different acids, the highest cellulose to glucose conversion rate of 316.7 g kg^{-1} of biomass was achieved with the pretreatment with nitric acid (results shown in figure 9). The lowest glucose yield of 221.3 g kg^{-1} was achieved with hydrochloric acid (Paper **IV**). These results were confirmed by statistical analysis which showed a significant difference ($p<0.05$) between glucose yields when using different acids in pretreatment phase.

For wheat straw samples that were pretreated with dilute sulphuric acid or dilute potassium hydroxide solution, two different approaches were used. Solid phase of half of the samples was rinsed with distilled water before enzymatic hydrolysis and the rest of the samples were not. Unrinsed samples pretreated with dilute sulphuric acid gave a glucose yield of 276.7 g kg^{-1} while samples that were rinsed before hydrolysis gave an average glucose yield of 267.3 g kg^{-1}, but this difference is statistically not significant ($p>0.05$). In the case of pretreatment with diluted KOH, the unrinsed samples gave a glucose yield of 221.7 g kg^{-1} while samples that were rinsed before hydrolysis gave a glucose yield of 267.5 g kg^{-1} (Paper **IV**) which is significantly different ($p<0.05$).
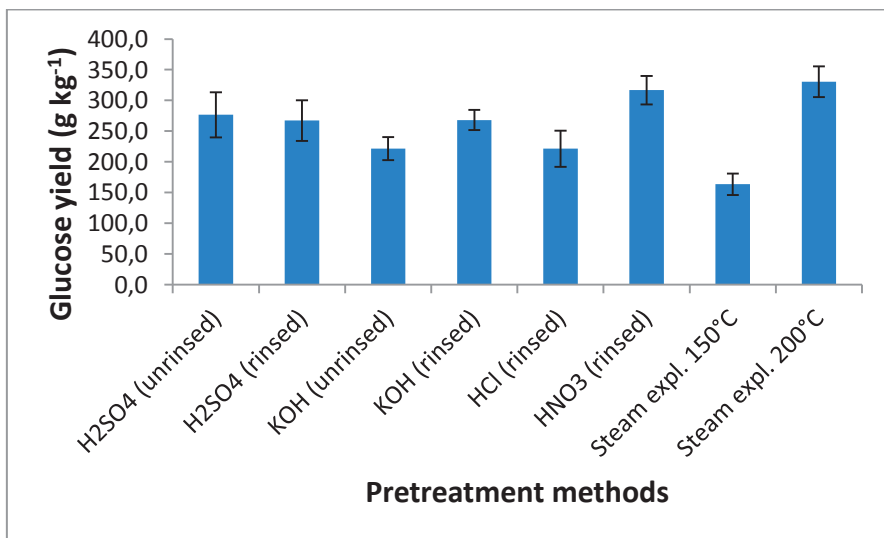

Figure 9. Glucose yields of wheat straw with different pretreatment methods.

Ethanol results of different pretreatment methods can be seen in figure 10. The rinsed samples pretreated with potassium hydroxide gave an ethanol yield of 104.3 g kg^{-1}. On the other hand, wheat straw samples pretreated with dilute nitric acid gave an ethanol yield of only 95.0 g kg^{-1} ($p<0.05$) regardless of the higher glucose yield (Paper **IV**).
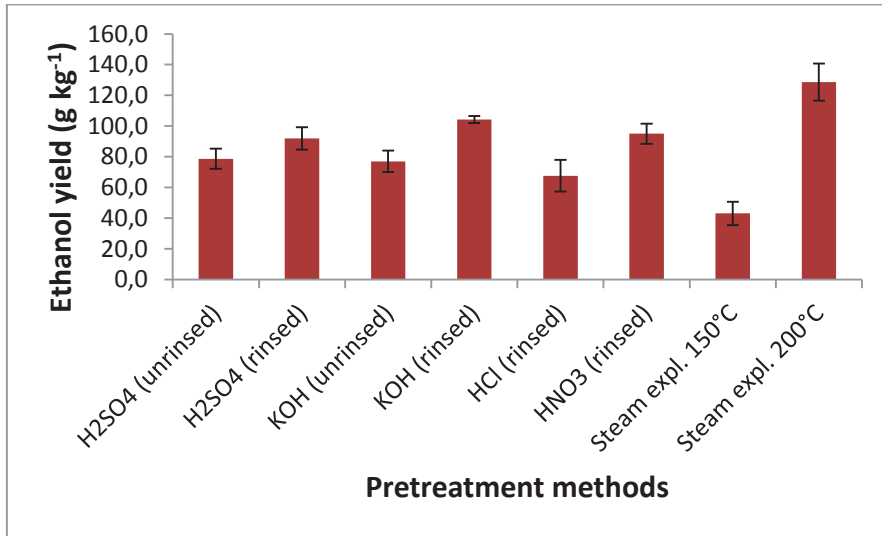

Figure 10. Ethanol yields of wheat straw with different pretreatment methods.

The effect of rinsing samples with distilled water was seen in the wheat straw samples pretreated with dilute potassium hydroxide as well. The rinsed wheat straw samples pretreated with KOH gave approximately 35.5% higher ethanol yield than the samples that were not rinsed (ethanol yields of 104.3 and 77.0 g kg^{-1} ($p<0.05$), respectively) (Paper **IV**). However rinsing did not have significant difference for samples pretreated with dilute sulphuric acid ($p>0.05$).

By far the highest and lowest glucose and ethanol yields were given by samples pretreated with steam explosion method at different temperatures. Samples pretreated with steam explosion at 200°C had glucose yield of 330.4 g kg^{-1} and an ethanol yield of 128.7 g kg^{-1}.

Steam explosion at 200°C had also the highest hydrolysis, fermentation and overall efficiencies of 71.1%, 76.4% and 54.3% (see table 4), while steam explosion at 150°C had by far the lowest efficiencies of 35.2%, 51.6% and 18.2%, respectively. From the rest of the compared

pretreatment methods, dilute nitric acid had high hydrolysis efficiency of 68.1%, but relatively lower fermentation efficiency of 59.2% while dilute potassium hydroxide pretreatment had the opposite effect with relatively lower hydrolysis efficiency of 57.7%, but higher fermentation efficiency of 76.3%.

Table 4. Hydrolysis and fermentation efficiencies of wheat straw with different pretreatment methods (GY- glucose yield; EY- ethanol yield; HE- hydrolysis efficiency; FE- fermentation efficiency; OE- overall efficiency).

Pretreatment method	GY (g kg^{-1})	EY (g kg^{-1})	HE (%)	FE (%)	OE (%)
H$_2$SO$_4$ (unrinsed)	276.7 ± 26.0	78.7 ± 4.7	59.5 ± 5.6	56.1 ± 6.5	33.2 ± 2.0
H$_2$SO$_4$ (rinsed)	267.3 ± 23.4	92.0 ± 5.1	57.5 ± 5.0	68.0 ± 6.3	38.8 ± 2.2
KOH (unrinsed)	221.7 ± 13.3	77.0 ± 4.9	47.7 ± 2.9	68.3 ± 5.2	32.5 ± 2.1
KOH (rinsed)	268.2 ± 10.3	104.3 ± 1.5	57.7 ± 2.2	76.3 ± 2.5	44.0 ± 0.6
HCl (rinsed)	221.3 ± 20.8	67.7 ± 7.3	47.6 ± 4.5	59.9 ± 7.9	28.6 ± 3.1
HNO$_3$ (rinsed)	316.7 ± 16.5	95.0 ± 4.6	68.1 ± 3.5	59.2 ± 4.6	40.1 ± 2.0
SE 150°C	163.6 ± 12.3	43.1 ± 5.4	35.2 ± 2.6	51.6 ± 7.0	18.2 ± 2.3
SE 200°C	330.4 ± 17.8	128.7 ± 8.5	71.1 ± 3.8	76.4 ± 6.4	54.3 ± 3.6

5.2.2. Nitrogen explosion pretreatment

5.2.2.1. Development of nitrogen explosion pretreatment system

In order to develop a pretreatment method that would be cost-effective and more efficient at moderate temperatures than steam explosion, a new nitrogen explosion pretreatment system was assembled (figure 6), together with the construction of modified reactor lid, seen on figure 11. The original reactor vessel with a volume of 2000 mL, together with reactor lid was made by Parr Instrument Company. Since the original lid did not allow carrying out explosive decompression, development of a new reactor lid became necessary. New reactor lid was made in the workshop of Estonian University of Life Sciences from re-melted stainless steel Mirrax 40 (modified AISI 420). Mirrax 40 has a carbon content of 0.21% and a chromium content of 13.5%, it has a high tensile strength of 1060 MPa at 200°C and a small thermal expansion coefficient of $10.6 \cdot 10^{-6}$ from 20 to

200°C (Uddeholm, 2014). Due to safety reasons, reactor pressure was limited to 60 bars and temperature to 220°C.
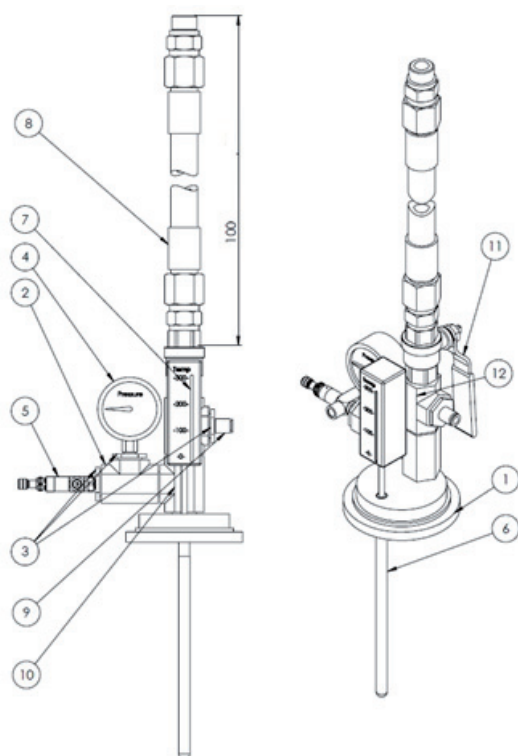

Figure 11. Design of the modified reactor lid for nitrogen explosion pretreatment system (1- reactor lid; 2, 3- pipe connections; 4- pressure gauge; 5- safety valve; 6- tube for temperature measurements; 7- thermocouple; 8- pressure release pipe; 9- gas connection link; 10, 12- triple connections; 11- pressure release valve).

5.2.2.2. Comparison of nitrogen explosion pretreatment and steam explosion pretreatment efficiencies

In order to assess the suitability of N_2 explosion pretreatment for cellulose conversion into sugars and bioethanol production, results from the N_2 explosion were compared to those from the steam explosion method as seen in figure 12 (Paper **V**). Floodplain meadow hay was used as a raw material in pretreatment comparison

experiments. Statistical ANOVA analysis confirmed the significant difference between the results of glucose yields pretreated with N_2 explosion and steam explosion methods ($p<0.05$) at all temperatures except at 190°C.
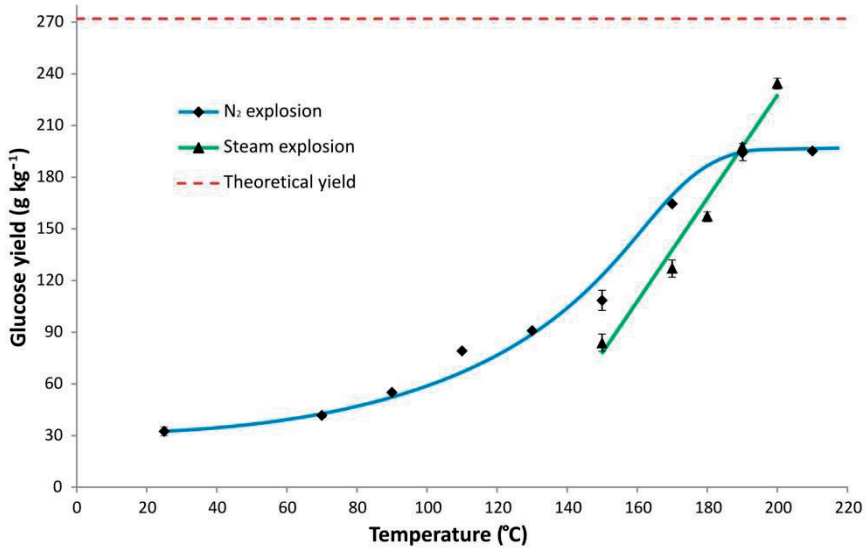

Figure 12. Glucose yield dependence of N_2 explosion and Steam explosion methods on the pretreatment temperature, where dashed line indicates the maximal theoretical glucose yield (Paper **V**).

Compared to steam explosion, the N_2 explosion pretreatment was more efficient at lower temperatures while steam explosion enables to gain higher glucose yields at temperatures over 190°C (Paper **V**).

Even though the highest cellulose to glucose conversion yield of 195.1 g kg^{-1} of biomass with the N_2 explosion pretreatment was achieved at a temperature of 210°C, the change in the yield between 190°C and 210°C is negligible. The overall highest glucose yield of 234.6 g kg^{-1} of biomass was achieved with steam explosion pretreatment at 200°C. The lowest glucose yield of 32.3 g kg^{-1} was achieved with N_2 explosion pretreatment at room temperature (25°C), which is approximately 6 times lower than the glucose yield achieved at 210°C (Paper **V**).

Ethanol yields are in correlation with glucose yields except in the nitrogen explosion results at 210°C, where regardless of high sugar concentration, active fermentation did not start.

As seen from the results in table 5, the glucose yield together with hydrolysis efficiency of N_2 explosion pretreatment rises in correlation with pretreatment temperature. The correlation can be best described by a fourth order polynomial curve. The difference of hydrolysis efficiencies at temperatures over 190°C is insignificant with hydrolysis efficiency of 71.3% at 190°C and 71.8% at 210°C, respectively (Paper **V**). The lowest hydrolysis efficiency of N_2 explosion pretreatment was observed at room temperature, which shows that explosive release of pressure alone, without thermal pretreatment, has little impact on plant cell wall structure. The highest overall efficiency of bioethanol production of 66.5% was seen with N_2 explosion pretreatment at 190°C.

The highest hydrolysis efficiency of 86.3% was achieved by steam explosion at 200°C, while the highest hydrolysis efficiency of 71.8% with explosive N_2 explosion pretreatment was achieved at 210°C (Paper **V**). Glucose yields with N_2 explosion pretreatment did not rise significantly at temperatures over 190°C due to biomass charring. The highest ethanol yields of 115.7 and 92.1 g kg^{-1} were obtained with steam explosion at 200°C and N_2 explosion at 190°C, respectively.

Table 5. Hydrolysis results at different pretreatment conditions (NE- N$_2$ explosion pretreatment at temperatures 25–210°C; SE- steam explosion pretreatment at temperatures 150–200°C).

Pretreatment method	GY g kg^{-1}	EY g kg^{-1}	HE %	FE %	OE %
NE25	32.3 ± 3.6	-	11.9 ± 0.5	-	-
NE70	41.5 ± 1.8	-	15.3 ± 0.3	-	-
NE90	54.9 ± 1.7	-	20.2 ± 0.4	-	-
NE110	79.0 ± 1.2	-	29.1 ± 0.4	-	-
NE130	90.8 ± 1.6	26.5 ± 0.2	33.4 ± 0.6	57.2 ± 0.7	19.1 ± 0.1
NE150	108.3 ± 10.4	36.8 ± 2.8	39.8 ± 4.6	56.5 ± 6.8	22.5 ± 2.0
NE170	164.4 ± 2.1	76.6 ± 2.6	60.5 ± 1.4	91.4 ± 3.4	55.3 ± 1.9
NE190	193.9 ± 4.6	92.1 ± 0.5	71.3 ± 3.6	93.1 ± 3.6	66.5 ± 0.3
NE210	195.1 ± 1.6	28.3 ± 0.2	71.8 ± 1.3	28.4 ± 1.4	20.4 ± 0.1
SE150	83.8 ± 6.5	19.8 ± 4.7	30.8 ± 2.2	46.3 ± 11.3	14.3 ± 3.4
SE170	127.1 ± 5.9	62.8 ± 3.6	46.7 ± 3.0	96.9 ± 6.3	45.3 ± 2.6
SE180	157.4 ± 3.0	70.7 ± 0.3	57.9 ± 1.9	88.1 ± 1.9	51.0 ± 0.2
SE190	197.7 ± 3.2	80.6 ± 7.0	72.8 ± 2.6	79.9 ± 7.4	58.2 ± 5.0
SE200	234.6 ± 3.8	115.7 ± 2.0	86.3 ± 3.6	96.7 ± 4.0	83.5 ± 1.4

5.2.2.3. Analysis of lignocellulosic material with scanning electron microscope (SEM)

In order to visualize the impact of N_2 explosion pretreatment on the surface of lignocellulosic material, a series of SEM images were taken from samples pretreated at different conditions. Analysed material was floodplain meadow hay. SEM images can be seen on figure 13.
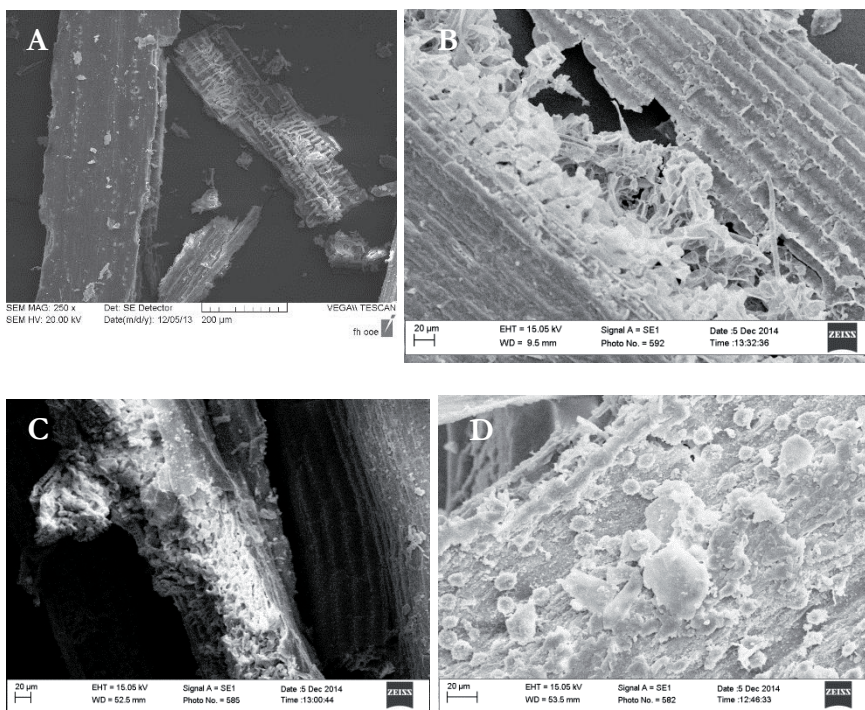

Figure 13. Comparison of scanning electron microscope (SEM) images of meadow hay samples - untreated (A) and N_2 exploded at different temperatures (B – 150°C, C – 170°C, and D – 200°C).

These images were compared to SEM images taken from samples pretreated with steam explosion at similar conditions, which can be seen in figure 14.
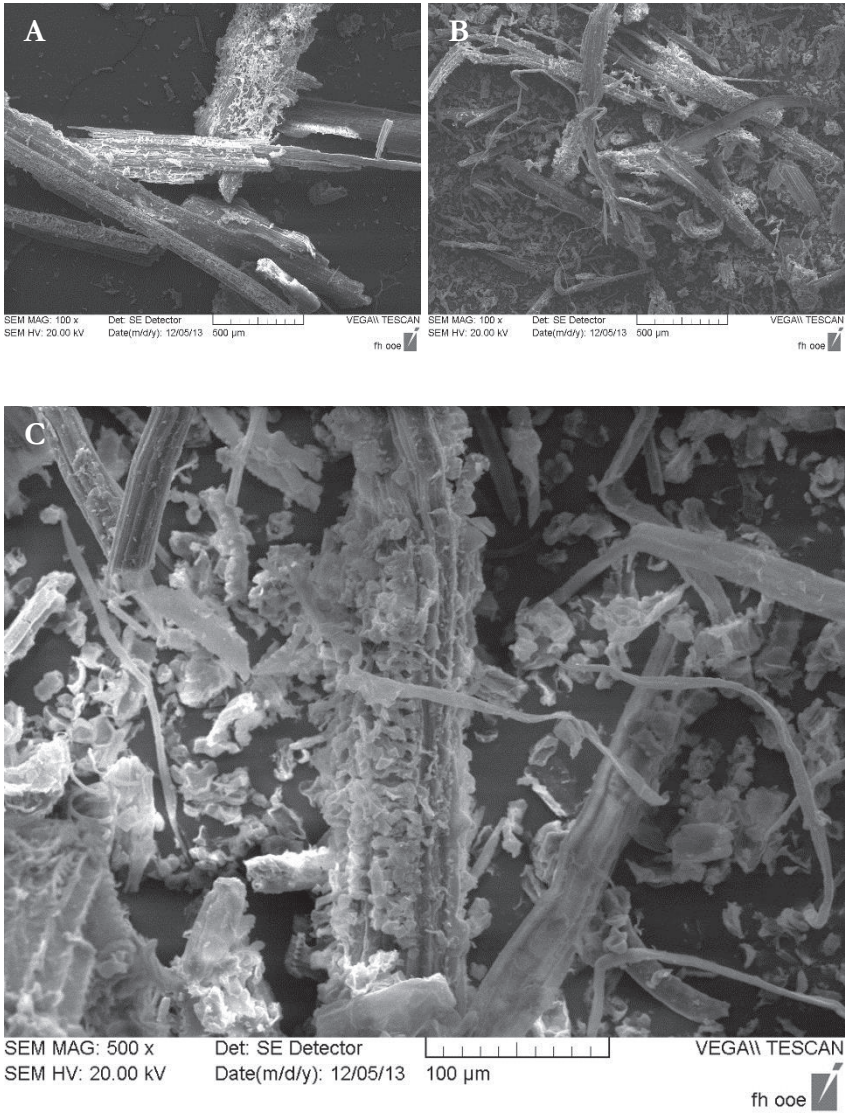
Figure 14. Comparison of scanning electron microscope (SEM) images of meadow hay samples steam exploded at different temperatures (A – 150°C, B – 170°C, and C – 200°C).

5.3. Bioethanol production potential in Estonia

Ethanol yields of steam explosion pretreatment at 200°C (wheat straw, floodplain meadow hay) and dilute acid pretreatment (hemp and Amur silver grass) were used to calculate potential annual bioethanol production in Estonia, see table 6. Wheat straw production in Estonia is approximately 385 000 tons per year (Statistics Estonia, 2014), but since some of the straw is used in farms and straw is also important for restoring soil conditions, then only 50% of wheat straw could be considered available for bioethanol production (Tarkalson et al., 2009; Weiser et al., 2014). From available wheat straw it is possible to produce 24 700 tons of bioethanol. Since the calorific value of ethanol is 29.7 MJ kg^{-1} which is approximately 37.7% lower than the calorific value of gasoline of 48.0 MJ kg^{-1}, then bioethanol from wheat straw corresponds approximately to 15 400 tons of gasoline or 17 500 tons of oil equivalent (toe). This bioethanol could replace about 7% share of the 234 000 tons of annual consumption of gasoline (Statistics Estonia, 2014). Barley is also widely grown in Estonia, but since barley straw was not experimentally analysed in this work, then annual bioethanol potential of barley straw was not included in results. Biomass collected from floodplain meadows could add another 11 500 tons of bioethanol (8155 toe) to the annual liquid biofuels production, which could replace approximately 3% of the annual gasoline consumption.

Table 6. Annual potential of bioethanol production in Estonia.

Energy crops	Acreage, ha	Available biomass, t	Annual bioethanol production, t
Wheat straw	124 200	192 500	24 700
Floodplain meadow hay	20 000	100 000	11 500
Hemp	83 000*	548 000	45 700
Amur silver grass	83 000*	871 500	67 200

*50% of the area of currently unused agricultural land suitable for growing energy crops.

Furthermore, Estonia has approximately 300 000 ha of agricultural land that is currently out of direct use. Putting this land back to use for energy crops production would not have a negative effect on food production. Much of this unused land is comprised of dispersedly situated small fields with an average size under 3 hectares. If we

consider, that only fields larger than 3 hectares will be used for growing energy crops, then the available resource of unused agricultural land would be 166 000 ha (Vohu, 2014).

At present, Amur silver grass and industrial hemp have acreage of less than 100 ha in Estonia, but since these are suitable energy crops for bioethanol production, the unused agricultural land could be efficiently used for growing Amur silver grass and/or industrial hemp. If half of the unused agricultural land would be used to grow industrial hemp and the other half to grow Amur silver grass, the biomass from these crops could be used to produce approximately 112 900 tons of bioethanol which corresponds to 80 080 toe.

6. DISCUSSION

6.1. Bioethanol production potential of different crops

Plant species with high cellulose content and high biomass yield have the highest potential for bioethanol production. Furthermore, wide overall growth area and non-competing with food production should be taken into account. From the results in table 1 it can be seen that crops with wide growth area, like wheat or floodplain meadow hay, have low above ground biomass yields. On the other hand, crops that produce high above ground biomass yields, like sunflower, Jerusalem artichokes, hemp or Amur silver grass, need to be cultivated and thus, would use arable land that otherwise could be used for growing food crops.

Bioethanol potential of different crops also depends on their growth location, weather conditions and vegetation phase (Papers **I, III**). Results indicate that plant species, which are native to other regions in the world, for example Amur silver grass, produce nearly 2.5 times less biomass in Estonian climatic and soil conditions than in its native region. This is confirmed by Bals et al. (2010) with the research of bioethanol production from switchgrass harvested in different seasons and locations. Unless experimental data from specific locations is taken into account, we may get a large overestimation when calculating bioethanol potential. In addition, harvesting season has an impact on plant biochemical composition and thus, on bioethanol yield (see table 3). As hemp matures, the cellulose and lignin contents in plant cells increase, which causes the stalk to wooden (Paper **III**). This corresponds well with earlier research done on biochemical composition of herbaceous crops like corn stover and wheat straw (Buranov and Mazza, 2008). Together with the increase of cellulose and lignin content in plant cells, the content of proteins and free sugars decreases, thus losing nutritional value, but also making the release of energy through cellulose conversion more difficult. When a plant has fully matured, adequate pretreatment method with harsh conditions is required to remove hemicellulose and lignin from plant cell walls to expose cellulose fibres. This explains why sunflower, which has one of the highest theoretical bioethanol potentials of 222 g m^{-2}, has very low experimental bioethanol yield of 49 g m^{-2} when inadequate conditions of dilute acid pretreatment are used (Paper **I**).

By taking into account the above ground biomass yield and experimental bioethanol yield, we can consider that the most suitable crops for bioethanol production in Estonia are Amur silver grass and hemp, because of their high above ground biomass and bioethanol yields. Wheat straw and floodplain meadow hay are also suitable crops for bioethanol production, because of their wide growth areas and the fact that they do not compete directly with food market for arable land (Papers **I, II**).

6.2. Comparison of different pretreatment methods

Different pretreatment methods and conditions were analysed in this work to investigate how they influence hydrolysis and fermentation efficiencies (Papers **II, IV, V**). The long term objective of this work was to develop a novel pretreatment method that would be cheap and relatively simple to use and would have high efficiency even at moderate pretreatment conditions.

6.2.1. Conventional pretreatment methods

Although the same acid concentrations were used, nitric acid pretreatment gave 30.1% higher glucose yield than the pretreatment with hydrochloric acid, with respective glucose yields of 316.7 and 221.3 g kg^{-1}. This shows that 1% (w/w) hydrochloric acid solution is unable to remove hemicellulose from the samples (Paper **IV**). Higher acid concentrations and longer pretreatment times could be used to overcome low hydrolysis rate, but this would make pretreatment with hydrochloric acid unfeasible compared to that with nitric acid or sulphuric acid. This corresponds well with literature (Markou et al., 2013) that nitric acid reacts faster with biomass and gives higher glucose and ethanol yields than sulphuric or hydrochloric acids. Sulphuric acid is favoured as a pretreatment agent in dilute acid pretreatment mainly because of its lower price compared to nitric and hydrochloric acids.

Results also confirm that rinsing samples with distilled water has an impact on hydrolysis and fermentation efficiencies if alkaline pretreatment is used, but rinsing does not have significant impact on

glucose and ethanol yields with dilute acid pretreatment. Approximately 21% lower glucose yield of unrinsed samples of alkaline pretreatment compared to the rinsed samples can be explained by the different viscosity of rinsed and unrinsed samples, and presence of dissolved lignin (Paper **IV**). Unrinsed samples were very viscous and thus, it was difficult to achieve uniform mixing of sample. Dissolved lignin in a solution is reported to inhibit enzyme activity. Dissolved lignin can non-specifically adsorb to enzyme molecules, thus making them inactive for hydrolysis process (Berlin et al., 2006).

As we can see from the results, samples pretreated with dilute acid have lower fermentation efficiencies than the ones pretreated with alkaline solution or with steam explosion (Papers **II, IV**). This can be explained by the formation of furfurals and organic acids during dilute acid pretreatment, which later inhibit fermentation process (Helle et al., 2003). Since during alkaline and steam explosion pretreatments these by-products are formed in very low quantities, the fermentation is more effective and more sugars are used for ethanol production rather than for the formation of acetic acid and other unwanted by-products.

Steam explosion at 200°C gave by far the highest glucose and ethanol yields together with highest overall efficiency of 54.3%. On the other hand, steam explosion at 150°C gave by far the lowest overall efficiency of 18.2%. This shows that steam explosion at high temperatures is very effective in exposing cellulose fibres and should be considered as the preferred pretreatment method for bioethanol production. However if high temperatures cannot be used in pretreatment phase, due to material or technological restrictions, other methods for pretreatment should be considered.

6.2.2. Nitrogen explosion pretreatment method

Steam explosion pretreatment is effective only at high temperatures and dilute acid or alkaline pretreatment methods are expensive because of the consumption of chemicals. In order to overcome these problems, a novel N_2 explosion pretreatment method was developed, which does not need additional chemicals, is cost-efficient and provides higher conversion efficiencies at moderate temperatures. Since steam explosion had the highest hydrolysis and fermentation

efficiencies in previous experiments, steam explosion was chosen as a benchmark for comparison with N_2 explosion pretreatment (Paper **V**).

SEM images show, that N_2 explosion pretreatment at 150°C has done damage to the sides of biomass particles where there have been previous mechanical injuries from milling, but most of the surface of biomass particles is intact. In case of N_2 explosion pretreatment at 200°C, there is extensive damage also to the biomass particle surface which is covered with pores, cavities and bubbles caused by expanding nitrogen gas. When we compare it to the images of steam exploded biomass at 200°C, we can see that steam explosion has done more structural damage to the plant cells through sheer mechanical force, instead of mainly damaging the cell wall surface but steam explosion has done even less damage to plant cell walls at 150°C than N_2 explosion pretreatment (Paper **V**).

Although steam explosion pretreatment enables to gain higher glucose and ethanol yields than N_2 explosion pretreatment at temperatures over 190°C, at pretreatment temperatures below 190°C N_2 explosion pretreatment has higher glucose and ethanol yields than steam explosion. For example, N_2 explosion pretreatment gave 13.8% higher hydrolysis efficiency at pretreatment temperature 170°C than steam explosion, at the same conditions. This can be explained by lower pressure difference in steam explosion at temperatures 150-190°C. For example, absolute pressures of saturated steam at 150°C and 180°C were 4.9 bar and 10.1 bar, respectively. Higher pressure difference and higher temperatures impact cell walls more and thus, yield more glucose. In case of N_2 explosion pretreatment, the explosive pressure release was always done at constant $P = 30$ bar. This means that compared to steam explosion, due to higher pressure at lower temperatures, more gaseous N_2 is able to penetrate plant cell walls and disrupt its structure.

In case of steam explosion pretreatment, correlation between glucose yield and temperature change is best described by linear curve in the experiment range, although at temperatures under 150°C or over 200°C, the results would fall outside the linear area. This can be explained with the fact that pressure of the saturated steam in the pretreatment vessel changes with temperature (Westman, 2008), but in

N$_2$ explosion pretreatment the explosive pressure release was done always at the same pressure (Paper **V**).

Although N$_2$ explosion pretreatment produced the highest glucose yields at temperatures of 190°C and 210°C, ethanol yields did not follow the same pattern. Ethanol yields rose in correlation with glucose yields until pretreatment temperature reached 190°C with maximum ethanol yield of 92.1 g kg^{-1}. When higher pretreatment temperatures were used, active fermentation did not start regardless of glucose concentration. That was probably caused by charring of biomass, which produced unwanted by-products that impeded yeast activity. The charring could be associated with the combination of high temperature, high dry matter content inside the reactor and lack of mixing. In the future research, it would be advantageous to decrease dry matter content or add mixing equipment to the reactor to avoid these problems.

These results show that N$_2$ explosion pretreatment can be used as an effective pretreatment method for bioethanol production at moderate temperatures (T<190°C) and the sugar conversion and ethanol yields are comparable to steam explosion results.

6.3. Bioethanol production potential in Estonia

In 2013, the annual consumption of liquid fuels in transportation sector in Estonia was 857 000 tons (933 000 toe), from which the share of gasoline was 234 000 tons, diesel 595 000 tons, and aviation fuel 28 000 tons (Statistics Estonia, 2014). Estonia has agreed to increase the share of renewable fuels in transportation sector to 10% by the year 2020, but in 2013 it was only 0.2%. One option to increase the share of liquid biofuels in transportation sector would be the wider use of lignocellulosic biomass for bioethanol production.

If available biomass of wheat straw and floodplain meadow hay together with biomass produced by growing industrial hemp and Amur silver grass on unused agricultural land would be used to produce bioethanol, it would yield annual bioethanol production of 149 100 tons (105 800 toe), which due to the lower calorific value of ethanol corresponds to 92 900 tons of gasoline. This bioethanol could replace

39.7% of gasoline consumption or up to 11.3% of overall liquid fuel consumption in Estonian transportation sector thus, fulfilling the target of 10% share of renewable fuels for 2020.

In future research, attention has to be paid also to the economic aspects of bioethanol production, which were not in the scope of current work.

7. CONCLUSIONS

This doctoral thesis focused on the investigation of the most suitable herbaceous biomass types and pretreatment methods for the utilization of lignocellulosic material for second generation bioethanol production in order to help to reach the target of 10% share of biofuels in transportation sector by 2020. The above ground biomass yields, experimental bioethanol yields and overall production efficiencies were taken into account in assessing the potential of second generation bioethanol production in Estonia. The main conclusions of this thesis are as follows:

1. Above ground biomass yield depends on crop type and growth location. Energy crops with the highest above ground biomass yields in Estonian climatic conditions were sunflower, Jerusalem artichoke and Amur silver grass. Biomass yields of these plant species were up to 2.5 times lower than in their native regions.

2. The highest overall efficiency of bioethanol production of 83.5% was provided by steam explosion method at pretreatment temperature of 200°C, but steam explosion method is ineffective at moderate temperatures (130–190°C).

3. A new pretreatment method was developed in this work. Patent application P201400050 of nitrogen explosion pretreatment method for disruption of cellular structure of biomass has been submitted to the Estonian Patent Office.

4. N_2 explosion pretreatment should be used in situations where moderate pretreatment conditions (130–190°C) are needed due to material or technological restrictions.

5. The most important properties of biomass feedstock for bioethanol production are high above ground biomass yield and high experimental bioethanol yield together with wide growth area. Also, it should be taken into consideration, that biomass used for bioethanol production should not compete with food market.

6. By taking into account the above mentioned properties, we can consider that the most suitable crops for bioethanol production in Estonia are Amur silver grass and hemp because of their high above ground biomass and bioethanol yields. In addition wheat straw and floodplain meadow hay should be considered because of their wide growth areas and the fact that they do not compete directly with food market for arable land.

7. If available biomass of wheat straw and floodplain meadow hay together with biomass of Amur silver grass and industrial hemp produced from unused agricultural lands would be used to produce bioethanol, it would make an annual bioethanol production of 149 100 tons, which could replace 39.7% of gasoline consumption or up to 11.3% of overall liquid fuel consumption in Estonian transportation sector.

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SUMMARY IN ESTONIAN

LIGNOTSELLULOOSSE BIOMASSI BIOKEEMILIST KOOSTIST MÕJUTAVAD TEGURID NING BIOKEEMILISE KOOSTISE MÕJU EELTÖÖTLUSMEETODI VALIKULE JA BIOETANOOLI TOOTLIKKUSELE

Seoses kasvavate keskkonnaprobleemide ning järsult kõikuvate kütusehindadega on viimasel aastakümnel toimunud maailmas pidev liikumine efektiivsemate ja väiksemate keskkonnamõjudega taastuvatest ressursidest toodetud kütuste kasutamisele. Euroopa Liit on seadnud eesmärgiks saavutada aastaks 2020 energiatootmises vähemalt 20%-ne taastuvenergia osakaal. Sealhulgas peab vähemalt 10% transpordisektoris kasutatud kütustest olema toodetud taastuvatest ressursidest.

Erinevate taastuvenergiaallikate seast peetakse transpordisektori jaoks kõige sobivamateks variantideks vedelaid biokütuseid ja biometaani, sest neid saab toota erinevatest taastuvatest toorainetest. Vedelate biokütuste hulgast on tõusnud eelistatuimaks valikuks lignotselluloosse bioetanooli tootmine, sest lignotselluloosne biomass kujutab endast kõige laiemalt levinud ülemaailmset ressurssi, millest on võimalik toota vedelaid biokütuseid.

Hoidmaks ära konflikti toidu- ja kütusetootmise vahel, tuleks bioetanooli tootmiseks kasutada ainult põllumajanduses tekkivaid kõrval saadusi ja jäätmeid, poollooduslikelt rohumaadelt kogutud biomassi ning energiakultuure, mille kasvatamiseks võetakse kasutusse hetkel kasutusest väljas olevaid põllumaid.

Töös uuriti Eesti klimaatilistes oludes kasvavate energiakultuuride ja poollooduslike rohumaade taimekoosluste potentsiaali bioetanooli tootmiseks, et kaasa aidata Eesti taastuvenergia eesmärkide täitmisele. Käesoleva doktoritöö peamisteks eesmärkideks olid:

1) Uurida erinevate taimeliikide biomassi juurdekasvu, biokeemilist koostist ja bioetanooli saagiseid (**I-V**).

2) Uurida taime kasvufaasi ja koristusaja mõju bioetanooli saagisele (**I, III**).

3) Leida valitud taimeliikidest bioetanooli tootmiseks optimaalne eeltöötlusmeetod ja biomassi eeltöötluse tingimused (**II, IV, V**).

4) Arendada bioetanooli tootmiseks välja eeltöötlusmeetod, mis oleks efektiivne ka mõõdukatel (130–190°C) temperatuuridel (**V**).

5) Arvutada välja potentsiaalne aastane bioetanooli toodang Eestis, vastavalt eksperimentaalselt leitud bioetanooli saagistele ning biomassi juurdekasvule (**I, II, V**).

Selleks uuriti järgmisi taimeliike ja –kooslusi: tööstuslik kanep (*Cannabis sativa L.*), päevalill (*Helianthus annuus L.*), maapirn (*Helianthus tuberosus L.*), amuuri siidpööris (*Miscanthus sacchariflorus L.*), energiahein Szarvasi, nisupõhk ning Emajõe luhalt kogutud luhahein. Proovid kuivatati ja jahvatati osakeste suuruseni 1 – 3 mm ning hoiustati toatemperatuuril $22 \pm 3°C$. Proovide kuivainesisaldus oli $90 \pm 1\%$. Proovide tselluloosi, hemitselluloosi ja ligniini sisaldust analüüsiti Eesti Maaülikooli taime biokeemia laboris.

Biomassi eeltöötlemisel kasutati happelist eeltöötlust, aluselist eeltöötlust, aurlõhkamist ning uudse meetodina lämmastiklõhkamist. Lämmastiklõhkamisel võeti 100 ± 0.1 g eelnevalt kuivatatud ja jahvatatud biomassi, mida leotati 500 ± 3 g destilleeritud vees ning seejärel sisestati survereaktorisse. Reaktor survestati gaasilise lämmastikuga $P = 30$ bar, kuumutati temperatuuridel $T = 70 – 210 \pm 3°C$ inkubatsiooniajaga $t = 15 – 120$ minutit. Soovitud temperatuuri saavutamisel jahutati reaktor alla 90°C ning teostati plahvatuslik rõhulangetus. Plahvatusliku rõhulangetamise teostamiseks valmistati Eesti Maaülikooli Tehnikainstituudis spetsiaalselt modifitseeritud reaktor. Doktoritöös väljatöötatud meetodi registreerimiseks on Eesti Patendiametisse esitatud patenditaotlus P201400050 „Lämmastiklõhkamismeetod biomassi rakustruktuuri lõhkumiseks“.

Töödeldud biomassi hüdrolüüsi ja fermentatsiooni katsed ning glükoosi ja etanooli analüüsid teostati Eesti Maaülikooli

Tehnikainstituudi kütuselaboris, välja arvatud aurlõhkamise katsed, mis toimusid Ülem–Austria Rakendusülikooli laborites.

Tulemustest selgus, et suurima biomassi juurdekasvuga taimeliigid on päevalill, maapirn ja amuuri siidpööris, kuid Eesti oludes on nende taimeliikide biomassi saagis kuni 2.5 korda väiksem kui nende liikide päritolu regioonides Aasias ja Põhja–Ameerikas. Bioetanooli tootlikkuse hindamisel tuleb lisaks biomassi juurdekasvule arvesse võtta ka taimeliikide biokeemilist koostist ja kasutatud eeltöötlusmeetodit. Kõrge hemitselluloosi ja ligniini sisaldus takistab ensüümide jõudmist tselluloosikiududeni ning nende lagundamist suhkru monomeerideks.

Erinevatest töös uuritud eeltöötlusmeetoditest osutus kõige efektiivsemaks aurlõhkamine temperatuuril 200°C, mis andis luhaheinast toodetud bioetanooli saagiseks 115.7 g kg^{-1} biomassi kuivaine kohta ning kombineeritud efektiivsuseks 83.5%. Samas ei sobi aurlõhkamine materjali töötlemiseks madalamatel temperatuuridel, näiteks luhaheina aurlõhkamine temperatuuril 150°C andis bioetanooli tootmise kombineeritud efektiivsuseks ainult 14.3%.

Kõrgemate efektiivsuste saavutamiseks eeltöötluse temperatuuridel alla 190°C arendati doktoritöö käigus välja uudne lämmastiklõhkamise meetod, mis sobib kasutamiseks olukordades, kus materjali või tehnoloogiliste piirangute tõttu ei saa biomassi töötlemiseks kasutada kõrgeid temperatuure.

Kõige tähtsamad omadused bioetanooli tootmise tooraine jaoks on suur biomassi juurdekasv, kõrge eksperimentaalne bioetanooli saagis koos suure kasvupinnaga. Lisaks tuleb arvesse võtta, et bioetanooli tootmiseks kasutatav tooraine ei tohiks konkureerida toidu tootmisega. Seetõttu leiti käesolevas töös, et Eesti oludes on kõige sobivamateks bioetanooli tootmise energiakultuurideks Amuuri siidpööris ja tööstuslik kanep. Lisaks on sobivad toorained ka nisupõhk, sest tegu on laia kasvupinnaga, kuid vähese kasutusega põllumajandusliku kõrval saadusega ning poollooduslikelt rohumaadelt kogutud biomass.

Kui saadaolev nisupõhk ja poollooduslike rohumaade biomass rakendada bioetanooli tootmiseks ning lisaks võtta kasutusse osa aktiivsest kasutusest väljas olevatest põllumajandusmaadest Amuuri siidpöörise ja tööstusliku kanepi kasvatamiseks, siis oleks Eestis

võimalik toota aastas ligikaudu 149 100 tonni bioetanooli (105 800 toe), mis võiks asendada 39.7% bensiini tarbimisest või kuni 11.3% üleüldisest vedelkütuste tarbimisest Eesti transpordisektoris.

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I

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Basis of Energy Crop Selection for Biofuel Production: Cellulose vs. Lignin

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Basis of Energy Crop Selection for Biofuel Production: Cellulose vs. Lignin

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Abstract

This paper investigates the suitability of Jerusalem artichoke (*Helianthus tuberosus* L.), fibre hemp (*Cannabis sativa* L.), energy sunflower (*Helianthus annuus* L.), Amur silver-grass (*Miscanthus sacchariflorus*), and energy grass cultivar (cv) Szarvasi-1 for biofuel production in Northern climatic conditions. Above ground biomass, bioethanol production yield, and methane production yield are used as indicators to assess the bio-energy potential of the culture. Results presented show that energy crops of Southern origin produce 30-70% less biomass than in the

origin region. Nonetheless, both perennial and annual energy crops produce high above ground biomass yields (660-1280 g m^{-2}) for Northern climatic conditions. Experimental results show that bioethanol yield is dependent on cellulose content of the biomass. The higher the cellulose content the higher the bioethanol yield. The biogas production on the other hand, depends on lignin content. The lower the lignin content the higher the biogas yield. Therefore, the selection of the energy crop for bioethanol production should be based on high cellulose content, while for biogas production it should rather be based on the low lignin content.

Keywords: biofuels, bioethanol, methane yield, cellulose content, lignin content, energy crop selection.

1. Introduction

The increasing industrialization and motorization of the world has led to a steep rise in the demand of petroleum-based fuels (Agrawal, 2007). Today fossil fuels take up to 80% of the primary energy consumed in the world, of which 58% is consumed by the transportation sector alone (Escobar et al., 2009). Among many energy alternatives, biofuels, hydrogen, natural gas and syngas (synthesis gas) may likely emerge as the four strategically important sustainable fuel sources in the future. Within these four, biofuels are the most environment-friendly energy source (Dennis et al., 2008). Biofuels are favorable choice due to their renewability, biodegradability, and generating acceptable quality exhaust gases (Bhatti et al., 2008). The key advantage of the utilization of renewable sources for the production of biofuels is the utilization of natural bio-resources (that are geographically more evenly distributed than fossil fuels) to

provide independence and security of energy supply (Hoekman, 2009; Tutt et al., 2012; Tutt et al., 2013).

In order to use the biomass as energy source to generate electricity, heat, or move vehicles, the biomass has to be converted into solid, liquid, or gaseous fuels. This conversion is generally achieved by two different processing routes: thermochemical (e.g., combustion, gasification, pyrolysis, and liquefaction) or biochemical (e.g., fermentation, anaerobic digestion) process (Flamos et al., 2011; Toor et al., 2011). Three types of energy can be produced from biomass - liquid fuels such as ethanol or pyrolysis oil, gaseous fuels such as biogas and electricity (Menon et al., 2012). Thermochemical processes like pyrolysis and gasification produce a synthesis gas (CO and H_2) from which different biofuels (diesel, aviation fuel, ethanol etc.) can be produced (Sims et al., 2010), however using hydrothermal liquefaction a liquid products, often called bio-oil or bio-crude can be produced (Toor et al., 2011). On the other hand biochemical processes apply enzymes or microorganisms to convert biomass biogas or to sugars prior to their fermentation to ethanol (Sims et al., 2010).

The two most prevalent liquid biofuels in transportation sector are ethanol and biodiesel (Flamos et al., 2010; Kreuger et al., 2011) however gaseous methane is promising candidate for renewable transportation fuel (Kreuger et al., 2011). Most of the ethanol is produced from sugars or easily degradable carbohydrates e.g. starch (Kreuger et al., 2011) however great attention has been placed to 2-nd generation biofuels produced from lignocellulosic feedstock (Sims et. al. 2010). Lignocellulosic feedstock is either non-edible residues of food crop production or non-edible whole plant biomass (Nigam and Singh 2011) which makes up the majority of the cheap

and abundant nonfood materials available from plants (Naik et al. 2010; Tutt et al., 2012). Utilizing agricultural residual and waste substrates as raw materials will minimize the potential conflict between food and fuel and also produces the bio-fertilizer and bio-pesticides. In addition, an abandoned agricultural or nutritionally depleted land can be used for the production of lignocellulosic (non-food) bio-energy crops to produce energy from cellulose and other cell wall polysaccharides (Campbell et al., 2008; DeBolt et al., 2009).

Three main components of any biomass are: hemicellulose, cellulose, and lignin. These generally cover 20-40, 40-60, and 10-25 % (by weight) of lignocellulosic biomass, respectively (McKendry, 2002). The studies have revealed that each group of substrates has a specific energetic potential that is linked to their chemical composition characteristics (Klimiuk, 2010).

Cellulose is the most abundant organic compound on Earth. It is the primary structural component of cell walls in biomass. Its content varies from 90% (by weight) in cotton to 33% in majority of other plants. Cellulose is a linear and highly ordered (often crystalline) polymer of cellobiose (D-glucopyranosyl-β-1,4-D-glucopyranose) (Higuchi 1997). It has high degree of polymerization (~10,000) and a large molecular weight (~500,000). It can be represented by the generic formula $(C_6H_{10}O_5)_n$,. This structure gives cellulose high strength, permitting it to provide the skeletal structure of most terrestrial biomass (Klass, 1998).

Hemicellulose is another major constituent of the cell walls of a plant. Unlike cellulose, hemicellulose has a random, amorphous structure with little strength. It is a group of carbohydrates with a branched chain structure and a lower degree of polymerization (~100-200). It can be represented by the generic formula $(C_5H_8O_4)_n$. There are significant variations in the

composition and structure of hemicellulose between different types of biomass. Most hemicelluloses, however, contain some simple sugar residues like d-xylose (the most common), d-glucose, d-galactose, l-arabinose, d-glucuronic acid, and d-mannose (Klass, 1998).

Lignin, the third important constituent of woody biomass, acts as a cement between fibres, but it is also present in the layers of the cell wall (especially the secondary cell-wall), forming, together with hemicelluloses, an amorphous matrix in which the cellulose fibrils are embedded and protected against biodegradation (Fengel et al., 1984). It is primarily three-dimensional polymer of 4-propenyl phenol, 4-propenyl-2-methoxy phenol, and 4-propenyl-2.5-dimethoxyl phenol (Diebold & Bridgewater, 1997). The dominant monomeric units in lignin are benzene rings. Therefore, it would generally have lower oxygen and higher carbon compared to cellulose or hemicelluloses (Basu, 2010).

Even though biomass as an alternative fuel source has been extensively researched, the possibilities of biomass usage in Northern climatic conditions are still to be thoroughly weighed. Northern climatic conditions present unique challenges due to the shorter growth period and harsh winter conditions. Therefore, the selection of lignocellulosic cultures with the highest bio-energy potential is a paramount.

Recently, fibre hemp and sunflower field experiments have been performed in Estonia. Preliminary results have shown that energy sunflower cultivation is quite promising (Noormets et al., 2010). Furthermore, field experiments with maize, the most popular energy crop in Europe, have also been performed in Estonia. Additionally, it has been proposed that some

alternative southern perennial and annual energy crops may have bio-energy potential in Nordic conditions.

The current study investigated the suitability of Jerusalem artichoke (*Helianthus tuberosus* L.), fibre hemp (*Cannabis sativa* L.), energy sunflower (*Helianthus annuus* L.), Amur silver-grass (*Miscanthus sacchariflorus*), and energy grass cultivar (cv) Szarvasi-1 for biofuel production, although these cultures are originally grown in southern climatic conditions. These potential energy crops produce high above-ground biomass in native habitats (Gunnarson et al., 1985; Zabaniotou et al., 2007; Bengtsson 2009; Tutt et al., 2013). Above ground biomass, bioethanol production yield, and methane production yield were used as indicators to assess the bio-energy potential of the culture.

2. Material and methods

2.1. Biomass

To investigate the suitability of different alternative crops in Estonian conditions for biofuel production, a plant field collection was established in 2010 at the Institute of Agricultural and Environmental Sciences of Estonian University of Life Sciences on Haplic Luvisol (Hypereutric) soil near Tartu, Estonia. The species grown in the collection field were: Jerusalem artichoke (*Helianthus tuberosus* L.), fibre hemp (*Cannabis sativa* L.) cv USO-31, energy sunflower (*Helianthus annuus* L.) cv Wielkopolski, Amur silver-grass (*Miscanthus sacchariflorus*), and energy grass cv Szarvasi-1. For Jerusalem artichoke, Amur silver-grass and energy grass cv

Szarvasi-1 this was the third year of vegetation, the seeds of fibre hemp and sunflower were sown by hand on 23 May 2012. The collection plot size was $5m^2$ without replications. Mineral N fertilizer (NH_4NO_3) was applied by hand on 9 June 2012 (100kg N ha^{-1}). During the vegetation period no pesticides were applied. The temperature and precipitation data of 2010 differed from the long term average. The year 2012 was a little more difficult for crop growth, with an average temperature higher than usual (in July the temperature was $5.1^{o}C$ higher than long-term average), the total precipitation during the growth period (May–August) being 277mm, i.e. 34mm lower than long-term average.

The height of the plants were determined before the harvest, samples for chemical analysis, bioethanol experiments, and methane amount determination were taken on October 12th 2012. In addition, sample of Jerusalem artichoke was taken also on September 10th 2012. The vegetation period in Estonian climatic conditions had finished by mid-October 2012; the average diurnal temperature was then below 5°C. By this time Amur silver-grass and Jerusalem artichoke were in flowering stage, the other crops were in matured stage.

The percentage of lignin, Acid Detergent Fiber (ADF), and Neutral Detergent Fiber (NDF) in the dry mass (DM) of all plant samples was determined at the Plant Biochemical Laboratory of Estonian University of Life Sciences (Tecator ASN 3430; AOAC, 1990; Van Soest et al., 1991). All samples were ground with Cutting Mill SM 100 comfort (Retsch GmbH) and then with Cutting Mill ZM 200 (Retsch GmbH).

2.2. Bioethanol

Dilute acid pretreatment followed by enzymatic hydrolysis was used for degradation of cellulose into glucose. This method is simple and uses cheap chemicals and mild operating conditions. Downside of method is lower conversion rate and possibility of inhibitory byproducts formation. Pretreatment is achieved by breaking the lignin seal and hemicellulose sheathing over cellulose and by disrupting the crystalline structure of cellulose (Dien et al., 2006); Yang et al. 2009).

Sample size was 75 g of dried and milled biomass (moisture content < 5%) to wich 750 ml of 1% H_2SO_4 solution was added. All samples were heated for t = 30 minutes at a temperature T = 150 ± 3ºC and a pressure of p = 5 bar. Samples were cooled below 50ºC and pH of the mixture was neutralized using $Ca(OH)_2$ to achieve pH = 4,5–5, because enzymes used in the hydrolysis are inactivated when pH < 4 or if pH > 6. Pretreatment was followed by enzymatic hydrolysis using enzyme complex Accellerase 1500. Enzymes were added to a sample in a proportion of 0,2 ml per g of biomass. Hydrolysis lasted for t = 48 hours under constant stirring and at temperature T = 50ºC. As a result most of the biomass (TS = 10%) was dissolved and the mixture turned into a brown liquid. After the hydrolysis, glucose concentration in all samples was measured reflectometrically (RQflex 10 reflectometer, Reflectoquant glucose & fructose test).

The trial data were processed using correlation, variance analyses (ANOVA) and descriptive statistics (Microsoft Exel and GraphPad Prism 5). The means are presented with their standard errors (±S.E.). Significance is presented with P<0.05 if not indicated otherwise. At least 3 parallel samples for ethanol analysis were used from every different biomass sort.

2.3. Biogas

The basic metabolic panel (BMP) test performed in this study was based on a modified version of the guidelines described by Owen et al., 1979. The experiment was carried out in triplicate in plasma bottles with an effective volume of 575 ml. Each replica consisted of 150 ml of inoculum and 0.3 gTS of substrate and 50 ml of distilled water. Total volume was 200 ml.

The inoculum used was collected from the anaerobic reactor of a wastewater treatment plant in Tallinn, Estonia. The inoculum was stored at room temperature, sieved through 2mm mesh and preincubated at mesophilic range (36°C) for 5 days before test setup to ensure activation and degasification of the sludge.

Energy crop samples were conditioned by drying and milling to achieve particle size of less than 1mm for homogenization of sample.

Oxygen from the headspace of the test bottles was flushed out by inducing a flow of N_2/CO_2 during 8 minutes before closing the bottles. Bottles were closed with butyl rubber stoppers and incubated during 77 days at mesophilic temperature (36°C). Gas samples were taken by connecting the test bottles to the gas chromatographer through a plastic tube attached to a needle. Gas production was analyzed by measuring the increase in pressure in the gas phase of test bottles using an absolute pressure transmitter (0-4 bar, SIEMENS). Gas composition of biogas samples were analyzed chromatographically using a gas chromatograph (Varian Inc., Model CP-4900) equipped with 2 columns: a Molsieve 5A Backflush heated column (20m x 0.53mm), and a PoraPLOT U heated column (10m x 0.53mm). Helium and argon were used as carrier gases in

columns 1 and 2, respectively. Total solids (TS) were analyzed according to method 1684 (U.S. Environmental Protection Agency - EPA). TS were determined after drying the sample at 105°C over night. pH was measured by a Sentron pH-meter 1001pH.

The data was processed using Pearson's correlation and descriptive statistics. The linear regression model of total methane yield (LCH$_4$ kgTS^{-1}) was performed using R version 2.12.2 (R Development Core Team, 2011).

3. Results and Discussion

3.1. Above ground biomass

Hemp and sunflower produce high above ground biomass yield and are little-intensive crops to cultivate (Gill & Vear, 1980; Zimdahl, 2004; Lauk et al., 2009). They suppress weeds well and are so fast growing that they do not require any kind of herbicide treatment (Gill & Vear, 1980; Zimdahl, 2004). Hemp generates about 10–15 t of biomass per hectare in dry matter and it is estimated that 1 ha of hemp absorbs about 2.5 t of CO_2, which results in a significant reduction of the greenhouse effect (Mankowski & Kolodziej, 2008). Sunflower, being one of the major oil crops cultivated worldwide, has the potential to become a biomass crop. Sunflower expresses a higher photosynthesis rate than other C_3 plants and grows rapidly to achieve a high biomass yield, up to 19 t ha^{-1} in dry matter in wide range of environments, from the equator to 55 N latitude (Hu, 2008). The mean above ground biomass in dry matter of hemp and sunflower in

Estonian conditions depending on N fertilization has been 3.1–10.9 and 7.7–13.5 t ha^{-1}, respectively (Lauk et al., 2009).

All species of perennial energy crops derived from Southern areas tolerated the winter cold in Northern climatic conditions, but the post-wintering development rate of these species was much lower than in the origin area. The development rate of plants depends on Growing Degree Days (GDD) accumulation rate. In Estonia the long term average GDD for vegetation period (May–September) is 1259 and by this time Amur silver-grass just started to flower, Jerusalem artichoke harvested in September did not flower yet, Jerusalem artichoke harvested in October did flower, only energy grass Szarvas-1 was in matured stage. The above ground biomass of perennial crops harvested once during vegetation period was 30–70% smaller than in the origin region (Table 2.).

A biomass can be classified on the basis of its relative proportion of cellulose, hemicellulose, and lignin (Table 1.). The chemical composition of plants changes during vegetation period. Additionally, the lignin content of above ground biomass is influenced by plants developmental stage during harvest time. Lower lignin content was determined in plant samples at lower developmental stage.

General content of measured carbohydrates in sunflower and Jerusalem artichoke was much lower than of other species in this collection. The low carbohydrates content of Jerusalem artichoke was caused by the lower developmental stage of plants at harvest time. Additionally, sunflower and Jerusalem artichoke are not lignocellulosic plants.

Approximate location for Table 1.

3.2. Ethanol production

Cellulose and lignin content of a plant varies in time. A plant culture harvested in summer tends to have higher cellulose content and give better glucose and ethanol yields than the same culture harvested in autumn (Bals et al., 2010). Results indicate that ethanol yield from different crops is directly proportional to cellulose content of a sample (Figure 1.). The higher the cellulose content the higher is the ethanol yield. Jerusalem artichoke (Sep and Oct) samples gave the lowest ethanol yield of 36.55 g kg^{-1} and 37.76 g kg^{-1} of biomass, respectively as these samples also had the lowest cellulose contents of 20.95% and 25.99%, respectively. The highest ethanol yield of 83.40 g kg^{-1} was obtained from hemp samples that had the highest cellulose concentration of 53.86%.

The only exception to this dependence was sunflower. No direct correlation was found between ethanol yield and lignin content of a sample, but samples with similar cellulose content and higher lignin and hemicellulose levels tend to give lower ethanol output. Sunflower has higher cellulose content than Jerusalem artichoke samples, but quite high lignin and ash levels as well. Lignin minimizes the accessibility of cellulose to enzymes, resulting in lower ethanol yield than expected.

Approximate location for Figure 1.

Combining of the ethanol production results with the above ground biomass results showed that ethanol yield per unit of area was significantly the highest for Amur silver-grass (Table 2.). Although cellulose content and ethanol yield per kilogram of biomass was highest for hemp, its

biomass production per unit area was significantly less than that of Amur silver-grass. Sunflower on the other hand, with the highest biomass yield per unit area showed very low hydrolysis efficiency (33.08%) and therefore, low ethanol yield. In this case low yield could be caused by inadequate pretreatment process. Dilute acid pretreatment at 150ºC for 30 minutes is not sufficient to remove high concentration of lignin from sample.

Approximate location for Table 2.

Jerusalem artichokes gave the highest hydrolysis efficiency values of 77.88% and 74.70%, regardless of harvesting time (Table 3.). This culture had the lowest cellulose content, but also the lowest lignin, hemicellulose and ash content. With few inhibiting factors, cellulose was easily accessible for degradation.

Approximate location for Table 3.

3.3. Biogas production

The accumulated methane production during 77 days of incubation at mesophilic temperature for perennial and annual energy crops was determined (Figure 2.). Lignocellulose has been found to be slowly and often incompletely degraded under anaerobic conditions (Lynd et al., 2002). The initial degradation rate of carbohydrates between perennial crops was the lowest in the biomass of Amur silver-grass (15 LCH_4 $kgTS^{-1}$ per day), but after the second day the degradation rate increased up to 19 LCH_4 $kgTS^{-1}$ per day and the high degradation rate (over 9 LCH_4 $kgTS^{-1}$ per day) continued for two weeks. The initial degradation rate of carbohydrates was the highest for the samples from perennial crop Jerusalem artichoke (105 LCH_4 $kgTS^{-1}$ per day), but after the

6th day the degradation rate decreased dramatically. In spite of different degradation rates, the total amount of CH_4 for these crops did not differ significantly (332 and 325 LCH_4 $kgTS^{-1}$, respectively).

Between the annual crops the initial degradation rate was the highest in the biomass of sunflower (63–64 LCH_4 $kgTS^{-1}$ per day), but after the 6th day the degradation rate decreased 3.5-5 times. The degradation behavior of other annual energy crops was similar (initial degradation rate 32-36 LCH_4 $kgTS^{-1}$ per day; after the 10th day the degradation rate decreased below 6 LCH_4 $kgTS^{-1}$ per day).

Approximate location for Figure 2.

High total methane yield was determined from samples of Amur silver-grass and Jerusalem artichoke (332 and 325 LCH_4 $kgTS^{-1}$, respectively), because these energy crops developed slower, their lignin content was smaller and therefore, development stage more suitable for methane production. These results suggest that proper harvest time should be considered for optimal biogas production (Amon et al., 2007; Oslaj et al., 2010).

Our study indicated methane yield dependence on biomass lignin content. The correlation between methane yield and lignin content was significantly negative (r= - 0.70; P<0.05). In comparison, hemicellulose and cellulose content did not influence methane yield production significantly.

4. Conclusions

Both annual and perennial energy crops in this study produced reasonably high above ground biomass yield for Northern climatic conditions, even though perennial crops harvested once produced 30-70% less biomass than in the origin region. Amur silver-grass and Jerusalem artichoke did not reach matured stage in Northern climatic conditions.

In ethanol production experiments the highest yield per kg of biomass was achieved from hemp sample. On the other hand, as hemp had the lowest above ground biomass production of all energy crops in the selection, the ethanol yield per unit of area was highest from Amur silver-grass. Sunflower, though with the highest above ground biomass yield, did not result in high ethanol yield, as sunflower had by far the lowest (33.08%) hydrolysis efficiency value.

Results showed that energy crops for ethanol production should be selected based on their cellulose content as ethanol yield per kg of biomass was directly proportional to the cellulose content in the energy crop, with the exception of sunflower. In the case of the latter, the low ethanol yield can be explained with high lignin and ash content that can interfere with the hydrolysis process.

In case of biogas, the energy crop selection should rather be based on lignin content than cellulose, as lignin content had negative proportional effect on biogas yield while cellulose content had no effect. Thus, Jerusalem artichoke, the least effective in ethanol production (36.55-37.76 g kg^{-1}), gave one of the highest biogas yields (325 L kg^{-1}), as it has both low lignin (5.05-5.70%) and cellulose (20.95-25.99%) contents. Hemp on the other hand has both high lignin

(8.76%) and cellulose (53.86%) contents and therefore, yielded best results in ethanol production (83.40 g kg^{-1}), but had below average output of biogas (295 L kg^{-1}).

Acknowledgements

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18

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20

Figure 1. Dependence of ethanol yield from cellulose content of different energy crops. Slope value of linear regression analysis was 1.601 ± 0.052 and $R^2 = 0.939$. Sunflower, as an outlier was not taken into account in linear regression analysis

Figure 2. Accumulated methane production (LCH$_4$ kgTS^{-1}) during 77 days of incubation at mesophilic temperature for perennial (black) and annual (grey) energy crops.

Table 1. Percentage of hemicellulose (HC), cellulose (CEL), lignin, total hydrocarbons and ash in biomass samples of different energy crops.

Energy crop	HC %	CEL %	Lignin %	TOTAL %	Ash %
Amur silver-grass.	30.15	42.00	7.00	**79.15**	5.37
Energy grass cv Szarvasi-1	27.33	37.85	9.65	**74.83**	7.01
Hemp	10.60	**53.86**	8.76	**73.22**	5.25
Sunflower	5.18	34.06	7.72	**46.96**	**9.78**
Jerusalem artichoke (Oct)	4,50	25.99	5.70	**36.19**	4.56
Jerusalem artichoke (Sep)	5.48	20.95	5.05	**31.48**	5.15

Table 2. Potential ethanol yield (g m^{-2}) of different energy crops in Estonian conditions

Energy crop	Biomass g m^{-2}	Ethanol g kg^{-1}	Ethanol g m^{-2}
Amur silver-grass	1050 ± 350	76.67	**81 ± 27**
Hemp	660 ± 70	**83.40**	55 ± 5
Sunflower	**1280 ± 730**	38.13	49 ± 28
En. grass Szarvasi-1	750 ± 250	61.19	46 ± 15
Jerusalem Artichoke (Oct)	1050 ± 250	37.76	40 ± 9
Jerusalem Artichoke (Sep)	1050 ± 250	36.55	38 ± 9

Table 3. Efficiencies of hydrolysis and fermentation stages of the ethanol production.

Energy crop	Hydrolysis efficiency %	Fermentation efficiency %
Jerusalem artichoke (Sep)	77.88	44.19
Jerusalem artichoke (Oct)	74.70	38.14
Energy grass cv Szarvasi-1	71.86	44.11
Amur silver-grass	59.80	59.86
Hemp	58.06	52.30
Sunfower	33.08	66.36

II

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Using steam explosion pretreatment method for bioethanol production from floodplain meadow hay

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Abstract. This article investigates influence of the steam explosion pretreatment method at different temperatures on sugar conversion rates and bioethanol production efficiencies from floodplain meadow hay. Floodplain meadow hay is used as a raw material, because these semi-natural grasslands need regular maintenance to preserve their high biodiversity. So far, this biomass has been largely unused, but it could provide a good feedstock for bioethanol production.

In this work, steam explosion pretreatment is used in combination with enzymatic hydrolysis. Effects of steam explosion pretreatment on the fibre content and cell wall structure are also studied. Results from fibre analysis show, that the floodplain meadow hay has very high lignin content of 24.16%, but relatively low cellulose content of 27.19%. Highest cellulose to glucose conversion rate of 234.6 g kg^{-1} and ethanol yield of 115.7 g kg^{-1} of biomass were achieved with the steam explosion pretreatment at 200°C. Scanning electron microscope (SEM) images show that pretreatment at 150°C does very little damage to plant cells, while steam explosion at 200°C disintegrates most of the plant cell walls and exposes cellulose fibres.

Key words: floodplain meadow hay, glucose, bioethanol, steam explosion.

INTRODUCTION

Biofuels are considered one of the most sustainable options in the foreseeable future for replacing fossil fuels in transportation sector (Nigam & Singh, 2011). The most widely produced biofuel in the world is bioethanol (Global Renewable Fuels Alliance, 2011). Most of the bioethanol is produced from corn or sugar cane, but the share of cellulosic bioethanol is rapidly increasing. The advantage of the cellulosic bioethanol, compared to traditional grain/sugar ethanol, is the fact that it is possible to use entire above-ground biomass of a plant for bioethanol production, thus enabling better efficiency and land use. Downside of the cellulosic bioethanol production is the need for large investments and sophisticated processing equipment (Stevens et al., 2004). In the future, the production of bioethanol is expected to include both, traditional grain/sugar crops and lignocellulosic materials (Demirbas, 2011).

Lignocellulosic raw materials represent the most abundant global resource for production of liquid biofuels (Lin & Tanaka, 2006; Talebnia et al., 2010). Since demand for biofuels has been increasing together with demand for food, a lot of

417

attention has been recently directed to the utilization of biomass from grasslands (McKendry, 2002; Heinsoo et al., 2010).

In Estonia, there is nearly 20,000 hectares of floodplain meadows with high biodiversity, that need regular maintenance (Kukk & Sammul, 2006). If these meadows are not maintained, they will quickly overgrow and lose much of their biodiversity. Floodplain meadows produce every year over 100,000 tons of biomass, which until now has found little use. In order to promote the management of semi-natural meadows, alternative uses for the biomass are required without changing the traditional management principals like harvesting time, avoidance of fertilizers and use of heavy equipment (Heinsoo et al., 2010). One alternative possibility is to use natural meadow hay as feedstock for bioethanol production and utilize it as a substitute for petrol. This would also help to promote rural development, reduce greenhouse gases and decrease the dependence from energy import (Demirbas, 2005).

Several different pretreatment methods for lignocellulosic biomass have been studied in the past. Among those, steam explosion and dilute acid pretreatment are the most widely used. In steam explosion pretreatment, lignocellulosic biomass is heated at elevated temperatures of 150–250°C with high pressure steam. After a few minutes of incubation time, the heated biomass is subjected to explosive decompression thereby, physically and chemically modifying the biomass (Cantarella et al., 2004). When biomass is exposed to high temperatures: hemicellulose is degraded, part of lignin is solubilized and cellulose binding is reduced. Under instantaneous decompression, superheated water flashes into steam and steam volume expands explosively. The impact force generated by flashing and volume expansion destroys cell structure. This tears materials into small pieces, cellulose fibre-bundles are separated from one another and their structures loosened thereby, re-distributing lignin and fully exposing cellulose (Chen & Zhang, 2012).

Dilute acid pretreatment uses cheap chemicals, mild operating conditions and is simple to perform. Downside of the dilute acid pretreatment method is a low conversion rate and formation of by-products that are inhibitory for the following fermentation process (Tutt et al., 2012; Tutt et al., 2013). Furthermore, most of the lignin remains intact. In the pretreatment with dilute acid, 0.5–1.5% sulphuric acid solution is added to the biomass to hydrolyse hemicellulose during 5–60 minutes at 130–200ºC. Higher temperatures require shorter time of pretreatment (Yang et al., 2009; Kim et al., 2011).

Aim of this research was to investigate bioethanol production from floodplain meadow hay and to compare at different conditions the influence of steam explosion pretreatment to the glucose and ethanol yield. Results from steam explosion pretreatment were also compared to the results of dilute acid pretreatment of floodplain meadow hay

MATERIALS AND METHODS

Biomass

Meadow hay samples were harvested in July, 2012, from the floodplains of Emajõgi. Samples were milled to a particle size of 1–3 mm and stored at a room temperature. Dry matter content of samples was 90.4%.

418

Pretreatment

Steam explosion pretreatment was used in this work. Sample size was 900 g of pre-dried and milled hay, which was soaked in 900 g of distilled water. Pretreatment was performed in a laboratory scale steam explosion system, seen in Fig. 1, at the University of Applied Sciences Upper–Austria. Steam explosion was carried out at temperatures T = 150–200°C and incubation times of 10–30 minutes. Pretreated material was then dried at temperature T = 40°C to a dry matter content of 95%.
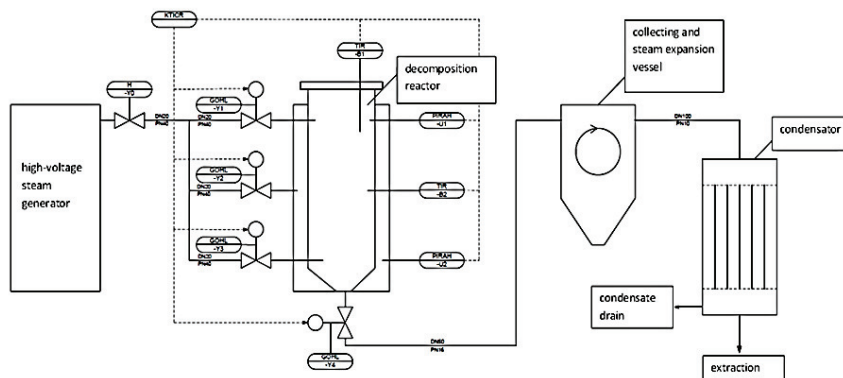
Figure 1. Laboratory scale steam explosion unit (Eisenhuber et al., 2013).

Pretreatment with dilute acid, followed by enzymatic hydrolysis, was used for the comparison tests. Size of the samples was 100 g of dried (DM 90.4%) and milled meadow hay to which 1,000 mL of 1% sulphuric acid solution was added. All samples were heated for t = 60 minutes at a temperature T = 130 ± 3°C and a pressure of p = 3 bar (Tutt et al., 2012; Tutt et al., 2013).

Hydrolysis and fermentation

Pretreatment phase was followed by enzymatic hydrolysis with enzyme complex Accellerase 1500. Enzyme mixture was added to the sample at a ratio of 0.3 mL per g of biomass. Hydrolysis of the pretreated material was carried out at 10% dry matter content in citrate buffer, c = 50 mmol L^{-1} and at pH = 5 (adjusted with NaOH). The samples were incubated for 72 h at 50°C in a shaking incubator at rotational speed of 2.5 s^{-1}.

Fermentation of glucose into ethanol was executed using yeast *Saccharomyces cerevisiae* in 1 litre bottles that were sealed with fermentation tubes. Volume of fermentation medium was 500 mL. Fermentation medium had a pH = 4.6 which was adjusted with H_2SO_4. Fermentation medium contained 100 mL hydrolysate, 2 mL $CaCl_2 \cdot 2H_2O$, 2 mL KH_2PO_4, 2 mL $MgSO_4 \cdot 7H_2O$, 0.44 g $(NH_4)_2HPO_4$. Yeast suspension (2 mL) was added to the solution and fermentation was carried out at 30°C for 120 hours.

419

Analysis

Dry matter content was analysed with a moisture analyser Ohaus MB 45. The fibre analysis (cellulose, hemicellulose and lignin) was performed according to the methods of Association of Official Analytical Chemists (AOAC 973.18) and methods by Tecator company (fibre determination using Tecator, Part No. 1000 1217, Serial No. 1706, U = 200 - 240 V, f = 50/60 Hz, P = 1,000 W). Fibre analysis results were also checked with acid hydrolysis. Acid hydrolysis was done according to the methods of National Renewable Energy Laboratory of USA for determination of structural carbohydrates and lignin in biomass (NREL, 2012).

Saccharides, organic acids, ethanol and furans in sample solutions were measured by high performance liquid chromatography (HPLC). The HPLC system Agilent Technologies 1200 Series with a Varian Metacarb 87 H column (300·7.8 mm) at 65°C, H_2SO_4 (c = 5 mmol L^{-1}) eluent and an isocratic flow rate of 0.8 mL min^{-1} was used. The signals were acquired with a refractive index (RI) and a UV–detector at 210 nm wavelength.

In order to compare the morphological structure of untreated and steam exploded raw material, scanning electron microscope (SEM) images were taken with a scanning electron microscope VEGA 2 LMU from Tescan.

Averaged results are used in figures and standard deviations are shown by vertical lines. Data was processed with programs Microsoft Excel and GraphPad Prism 5.

RESULTS AND DISCUSSION

Bioethanol production from natural meadow hay using steam explosion pretreatment was studied in this work. Glucose and ethanol results were compared with those from dilute acid pretreatment method that had been used previously for meadow hay pretreatment.

Table 1. Cellulose, hemicellulose, lignin and dry matter content of meadow hay samples (HS150– steam exploded hay at 150°C)

	Cellulose (%)	STD_2 (%)	Hemicellulo se (%)	STD_2 (%)	Lignin (%)	STD_2 (%)	Dry matter (%)
Hay (untreated)	27.19	0.69	29.15	0.48	24.16	0.29	90.43
HS150	32.66	0.23	26.51	0.15	26.49	0.31	93.46
HS170	33.42	1.11	25.98	0.38	28.82	0.12	93.96
HS180	33.87	0.36	24.63	1.02	29.05	0.09	95.20
HS200	35.07	0.36	15.27	0.48	33.71	0.17	95.83

Results from fibre analysis show that natural meadow hay, which was cut in the middle of July, has fully matured and therefore, has very high lignin content of 24.16%, but relatively low cellulose content of 27.19% in the untreated sample, see Table 1. Meadow hay also has relatively high hemicellulose content of 29.15%, which makes it difficult for enzyme molecules to reach cellulose fibres and degrade these into glucose without using adequate pretreatment conditions. High content of lignin in plant fibres leads to a creation of protective barrier that prevents plant cell destruction by

420

fungi and bacteria. For the conversion of biomass to biofuel, the cellulose and hemicellulose must be broken down into their corresponding monomers (Kumar et al., 2009).

Steam explosion pretreatment disrupts the structure of plant cell walls and removes hemicellulose, but it dissolves only a fraction of lignin. Approximately 95% of lignin remains in an insoluble form. Since no chemicals are used in the pretreatment phase, the steam explosion method requires high temperatures to effectively dissolve hemicellulose, as seen in Fig. 2. Effectiveness of steam explosion increases rapidly at temperatures over 180°C. Steam explosion at 200°C lowers hemicellulose content of meadow hay sample to 15.27%, compared to the 29.15% in the untreated sample.
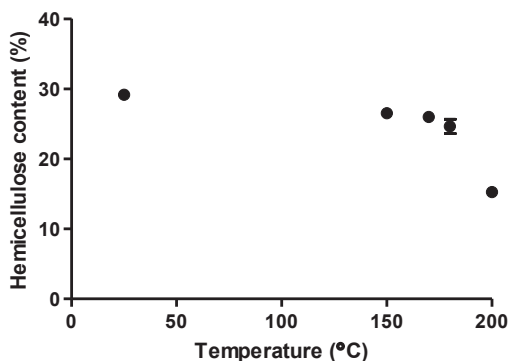
Figure 2. The correlation of hemicellulose content in meadow hay samples, from steam explosion pretreatment temperatures.

Scanning electron microscope images also confirm that steam explosion at 200°C is very effective in disrupting the plant cell wall structure, while temperatures under 180°C clearly seem to be inadequate, see Fig. 3. SEM images show that steam explosion at 150°C has done very little damage to cell walls compared to the untreated sample, while steam explosion at 200°C has destroyed most of the cell walls and exposed cellulose fibres.

Hydrolysis results show that the highest cellulose to glucose conversion rate of 234.6 g kg^{-1} of biomass was achieved with the steam explosion pretreatment at 200°C (results shown in Fig. 4). This shows that steam explosion at 200°C removes most of the hemicellulose from the sample and leaves the cellulose fibres easily accessible for enzymes. By far the lowest glucose yield of 83.8 g kg^{-1} was achieved by steam explosion pretreatment at 150°C. Although the pretreatment temperature was only 50 degrees lower, the glucose yield was 2.8 times smaller than the results achieved with pretreatment at 200°C temperatures. This shows that steam explosion conditions at 150°C are not effective enough to remove hemicellulose from the samples.

421

Figure 3. Comparison of scanning electron microscope (SEM) images of meadow hay samples – untreated (A) and steam exploded at different temperatures (B – 150°C, C – 170°C, and D – 200°C).

Figure 4. Influence of different pretreatment conditions on the glucose and ethanol yields from floodplain meadow hay samples.
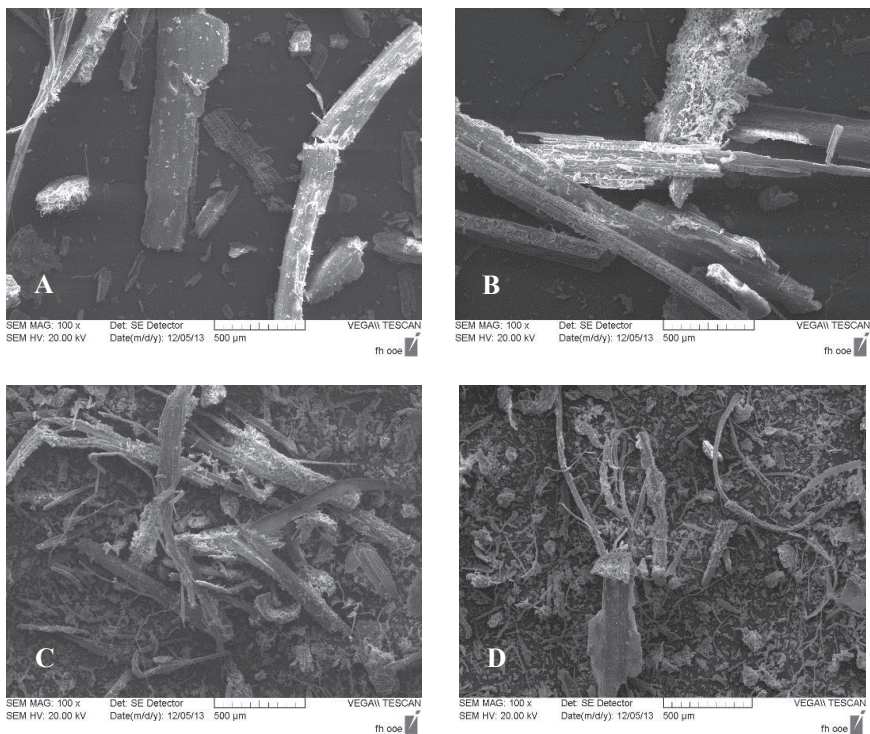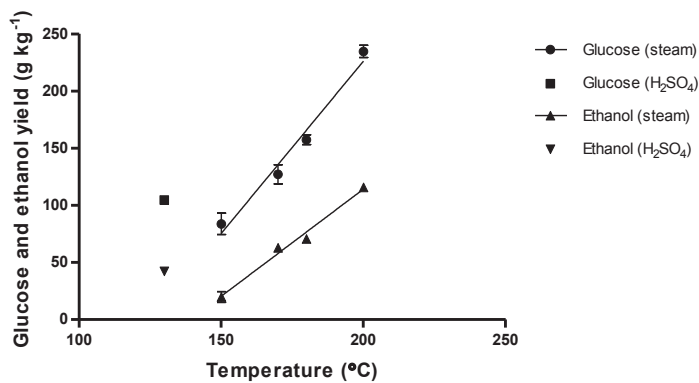
422

111

The same correlation was also seen between steam explosion temperature and ethanol yield. The highest ethanol yield of 115.7 g kg^{-1} was achieved by pretreatment at 200°C and the lowest ethanol yield of 19.8 g kg^{-1} was achieved by pretreatment at 150°C.

Table 2. Hydrolysis and fermentation efficiencies at different pretreatment conditions (HS-steam explosion pretreatment at temperatures 150–200°C; HD- dilute acid pretreatment at 130°C)

Pretreatment method	Glucose yield g kg^{-1}	Ethanol yield g kg^{-1}	Hydrolysis efficiency %	Fermentation efficiency %
HS150	83.8	19.8	30.8	46.2
HS170	127.1	62.8	46.7	96.9
HS180	157.4	70.7	57.9	88.1
HS200	234.6	115.7	86.3	96.7
HD130	115.2	41.1	42.4	69.8

The floodplain meadow hay pretreated with dilute acid gave glucose and ethanol yields of 115.2 g kg^{-1} and 41.1 g kg^{-1}, respectively. This shows that pretreatment of meadow hay with dilute acid is more effective than steam explosion at 150°C, but less effective than steam explosion pretreatment at temperatures over 170°C. Hydrolysis efficiencies at different pretreatment conditions are given in Table 2. The highest hydrolysis efficiency of 86.3% and one of the highest fermentation efficiencies of 96.7% was achieved by steam explosion at 200°C.

CONCLUSIONS

Results from fibre analysis show, that natural meadow hay has very high lignin content of 24.16%, but relatively low cellulose content of 27.19%. This means that it is difficult for enzyme molecules to reach cellulose fibres and degrade these into glucose without using high temperature pretreatment conditions.

Highest cellulose to glucose conversion rate of 234.6 g kg^{-1} and ethanol yield of 115.7 g kg^{-1} of biomass were achieved with the steam explosion pretreatment at 200°C. The lowest glucose yield of 83.6 g kg^{-1} and ethanol yield of 19.8 g kg^{-1} were given by samples pretreated with steam explosion at 150°C. These results were confirmed by scanning electron microscope images which show that pretreatment at 150°C does very little damage to plant cell walls. Fully matured meadow hay is quite durable to steam explosion pretreatment thus, higher temperatures and harsher conditions, preferably 200°C have to be used.

Although floodplain meadow hay has relatively low cellulose content and high lignin content, it is suitable raw material for bioethanol production. Floodplain meadows produce over 100,000 tons of biomass per year and if steam explosion pretreatment at 200°C, followed by enzymatic hydrolysis, is used, then it would be possible to produce approximately 11,570 tons of bioethanol from this biomass.

423

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424

III

Tutt, M., Kikas, T., Olt, J. 2013.
Influence of harvesting time on biochemical composition and glucose
yield from hemp.
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Influence of harvesting time on biochemical composition and glucose yield from hemp

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Abstract. This article investigates the influence of different harvesting times of hemp samples on their biochemical composition and glucose conversion yield. Samples were harvested from experimental fields of the Estonian University of Life Sciences from July to September in 2011. Dilute sulfuric acid solution was used for pretreatment in combination with enzymatic hydrolysis. Results indicate that the highest glucose conversion rate of 204.1 g kg^{-1} of dry matter of biomass was achieved by samples harvested on the 18th of August. The lowest glucose yield of 170.3 g kg^{-1} was achieved by samples harvested on 25th of August, which also had a very low hydrolysis efficiency of 46.9%. Biochemical composition and glucose conversion efficiencies of samples vary in time. Samples harvested in September have higher cellulose and lignin content than samples harvested in July. However, glucose conversion efficiencies decrease significantly in later samples. Average hydrolysis efficiency was 51.4%.

Key words: *glucose, dilute acid pretreatment, cellulose, hemp.*

INTRODUCTION

The increasing industrialisation and motorisation of the world has led to a steep rise in the demand for petroleum-based fuels (Agrawal, 2007). Today fossil fuels take up to 80% of the primary energy consumed in the world, of which 58% is consumed by the transportation sector alone (Escobar et al., 2009). The transportation sector is almost fully dependent on liquid fuels such as petrol and diesel. Continuously increasing oil prices have raised more support for the use of renewable energies. The key advantage of the utilisation of renewable sources for the production of biofuels is the use of natural bio-resources (that are geographically more evenly distributed than fossil fuels) to provide an independent and secure energy supply. Among biofuels, ethanol is one of the most appealing choices, because it can be blended with petrol or used in its pure form in modified engines (Hahn-Hagerdal et al., 2006; Tan et al., 2008). The dominating substrates used for ethanol production today are either pure sugars (sucrose from sugarcane) or easily degradable carbohydrates (starch from cereals or corn). A shift to lignocellulosic plant material is making the utilisation of other crops possible and will enable the production of transportation fuel from herbaceous biomass, corn stalks and straw (Kreuger et al., 2011). Utilising agricultural residual and waste substrates as raw materials for fuel ethanol production will also minimise the potential conflict between food and fuel.

Cellulosic ethanol production is a complex process compared to the first generation grain or sugarcane ethanol production. Firstly, it is necessary to break the

lignin seal and hemicellulose sheathing over cellulose, and disrupt the crystalline structure of cellulose. Only then is it possible to degrade the cellulose in the biomass to sugar monomers. This disruption is achieved by the pretreatment process that is usually followed by enzymatic hydrolysis (Dwivedi et al., 2009; Kim et al., 2011).

The energy crop investigated in this study was industrial hemp. Hemp has several features that make it a suitable feedstock for cellulosic ethanol production. Firstly, it yields high biomass per hectare and adapts easily to different climate conditions. Secondly, the cellulose content of hemp is usually between 35–55%. Hemp fibres have many industrial applications, for example it is used in composite materials, in textile or pulp and paper production (Gonzales-Garcia et al., 2012). Hemp has also been reported as a good solid fuel for combustion. The high cellulose content and above ground biomass yield per hectare makes hemp a suitable crop for bioethanol production (Sipos et al., 2010). Since hemp also has quite a high content of lignin, it is necessary to use hydrothermal pretreatment before enzymatic hydrolysis to make cellulose fibres more easily accessible for enzymes.

Pretreatment with a dilute acid was used in the current research. Dilute acid pretreatment has been the most widely used method for pretreatment of the lignocellulosic material. This method uses cheap chemicals, mild operating conditions and is simple to perform. The downside of the dilute acid pretreatment method is a low conversion rate and formation of byproducts that are inhibitory for the following fermentation process. For the pretreatment with dilute acid, 0.5–1.5% acid solution is added to the biomass to hydrolyse hemicellulose during 5–60 minutes at 130–200 °C. Higher temperatures require a shorter time of pretreatment (Dien et al. 2006; Yang et al., 2009).

The pretreatment process is usually followed by enzymatic hydrolysis to convert the cellulose fibres and hemicellulose to fermentable sugars and fermentation to convert sugars to ethanol. Hydrolysis is carried out by different cellulases which are produced by lignocellulose degrading bacteria or fungi, for example *Trichoderma reesei*. The main factors that affect the hydrolysis rate of cellulose are accessibility of cellulose fibres to enzymes, crystallinity of cellulose and hemicellulose, and lignin content (Sun et al., 2002; Kim et al., 2011). Presence of lignin and hemicellulose makes the access of enzymes to cellulose fibres difficult. Therefore, removal of lignin and hemicellulose as well as increase in porosity during the pretreatment process increases the hydrolysis rate significantly (Dwivedi et al., 2009). At the same time, presence of dissolved lignin can also inhibit the hydrolysis, so that not all of the accessible cellulose is converted to sugars. Enzymatic hydrolysis can be carried out with total solid loadings up to 20%. If solid loading is higher than that, the constant stirring and equal distribution of enzymes in the mixture becomes difficult to achieve.

The aim of this research was to assess the potential of hemp as a feedstock for bioethanol production and investigate how different harvesting times influence it.

MATERIALS AND METHODS

Biomass

Industrial hemp was chosen as a raw material in this work, because it has a high biomass yield and high cellulose content, which makes it suitable for ethanol production. Furthermore, hemp does not compete directly with the food market. Hemp

216

samples were harvested from July to September 2012, from the experimental fields of the Estonian University of Life Sciences. Hemicellulose, cellulose and lignin contents of aboveground biomass samples were determined in the Laboratory of Plant Biochemistry of the Estonian University of Life Sciences (see table 1). Standard methods of the Association of Official Analytical Chemists (AOAC 973.18) and methods by Tecator Company (fibre determination using the Fibertec M&I systems) were used in the analysis. Samples were milled to a particle size of 1–3 mm and dried. Dry matter content was $90 \pm 3\%$.

Table 1. Hemicellulose, cellulose and lignin contents in dry mass of hemp samples harvested at different times

Harvesting time	Hemicellulose %	Cellulose %	Lignin %
28th July	10.54	33.06	6.48
4th August	14.34	32.98	6.29
11th August	10.90	36.76	7.37
18th August	9.36	39.31	7.60
25th August	9.20	36.34	7.98
1st September	10.04	37.84	8.16
8th September	8.81	43.30	8.15

Methods

Pretreatment with dilute acid, followed by enzymatic hydrolysis, was used in this work. Size of the samples was 100 g of dried (DM $90 \pm 3\%$) and milled hemp to which 1,000 mL of 1% sulfuric acid solution was added. All samples were heated for t = 60 minutes at a temperature $T = 130 \pm 3°C$ and a pressure of p = 3 bar. As enzymes are inactivated when temperature is $T > 70 °C$ or $4 > pH > 7$, the sample was cooled to a temperature below 50 °C and K_2CO_3 was added to neutralise the pH. Pretreatment was followed by enzymatic hydrolysis with the enzyme complex Accellerase 1,500. Enzyme mixture was added to the sample at a ratio of 0.3 mL per g of biomass. Hydrolysis lasted for t = 24 hours under constant stirring and at a temperature $T = 50 °C$. After the hydrolysis process, glucose concentration in all of the samples was measured reflectometrically using RQflex 10 reflectometer and Reflectoquant glucose & fructose test. D–glucose and D–fructose are converted into D–glucose–6–phosphate. This is oxidised by NAD under the catalytic effect of glucose–6–phosphate dehydrogenase to gluconate–6–phosphate. In the presence of diaphorase, the NADH formed in the process reduces a tetrazolium salt to a blue formazan that is then determined reflectometrically.

At least three parallel samples were analysed from each harvesting time. Averaged results are used in figures and deviations are shown by vertical lines. Data was processed with programs Microsoft Excel and GraphPad Prism 5.

RESULTS AND DISCUSSION

Results confirm that the cellulose and lignin content of the hemp biomass rises with maturity (Fig. 1). Samples harvested at the end of July and beginning of August have the lowest cellulose and lignin content of 32.98 and 6.29%, while the latest harvest, cut on September 8th, has one of the highest cellulose and lignin contents of

217

43.30 and 8.15%, respectively. As hemp matures, the cellulose and lignin contents in plant cells increase, which causes the stalk to harden. This corresponds well with other research done on cellulose and lignin content in herbaceous crops like corn stover and wheat straw (Buranov and Mazza, 2008). However, the hydrolysis efficiency decreases in samples of later harvests (Figs 1, 2). This can be explained by the increasing lignin content of the samples. The higher the lignin content, the more difficult it is for enzymes to reach cellulose fibres and convert them to glucose monomers. The highest hydrolysis efficiency of 59.1% was achieved in the hemp sample harvested on August 4th, which also had the lowest lignin content of 6.29%. The lowest hydrolysis efficiency of 46.4% was achieved in the hemp sample harvested on September 8th. Although it had the highest cellulose content, it also had one of the highest lignin contents. In order to achieve better hydrolysis efficiencies from late harvest hemp samples, longer pretreatment times or higher temperatures should be used. Furthermore, the pretreatment with a dilute base could be used to break the lignin seal of the fibres as the dilute acid pretreatment affects mainly hemicellulose sheathing. Average hydrolysis efficiency for all samples was 51.4%.

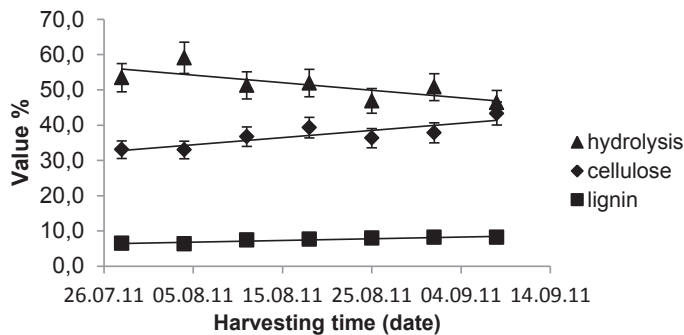**Figure 1.** Correlation of cellulose/lignin contents and hydrolysis efficiencies of hemp samples with harvesting times.
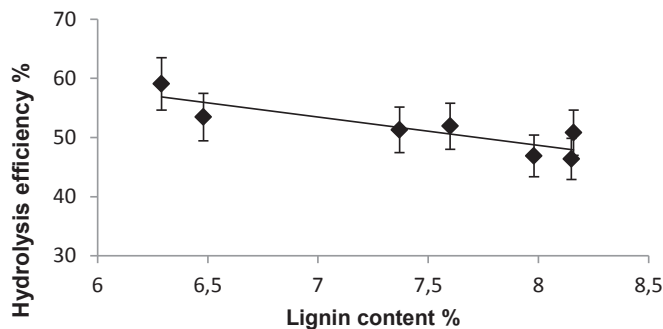

Figure 2. Dependence of hydrolysis efficiencies from the lignin content of the hemp samples with different harvesting times.

Glucose yield from the enzymatic hydrolysis was analysed for all hemp samples and the results were correlated with the harvesting time and cellulose content to assess the potential of hemp as a feedstock for bioethanol production. The corresponding results are shown in figure 3. Linear correlation was observed between glucose yield and cellulose content, as only cellulose fibres in lignocellulosic material are converted to glucose monomers. However, the highest glucose yield of 204.1 g per kg of dry matter was achieved from hemp samples harvested on August 18th and not from samples harvested on September 8th which had the highest cellulose content. This can be explained by the increasing lignin content and correspondingly decreasing hydrolysis efficiency of glucose conversion in hemp samples. From the point of glucose conversion and bioethanol production, the best harvesting time of hemp is in the middle of August when the plant has almost reached its maximum growth, but has not yet fully matured.
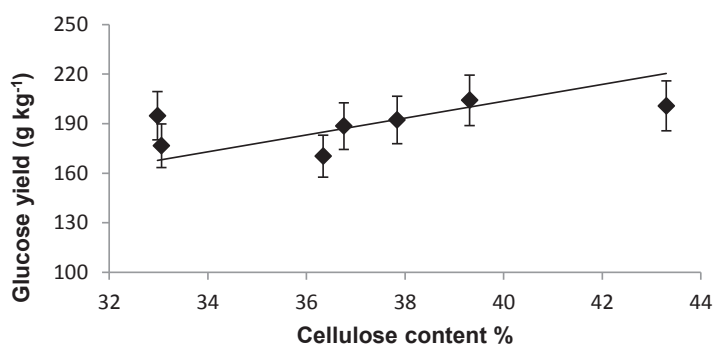
Figure 3. Dependence of glucose yield from cellulose content.

CONCLUSIONS

Aim of this research was to assess the potential of hemp as a feedstock for bioethanol production and investigate how different harvesting times influence it. Results confirm that cellulose and lignin content of a sample rises in time. Samples harvested at the end of July and beginning of August have the lowest cellulose and lignin content of 32.98 and 6.29%, while the latest harvest, cut on September 8th, have one of the highest cellulose and lignin contents of 43.30 and 8.15%, respectively. The hydrolysis efficiency decreases in samples of later harvests. Average efficiency for all samples was 51.4%. The highest glucose yield of 204.1 g per kg of dry matter was given by hemp samples harvested on August 18th and not by samples from later harvests which had higher cellulose contents. In conclusion, hemp is a good feedstock for bioethanol production, because of its high glucose yield and high above ground biomass yield. The best harvesting time of hemp for bioethanol production is in the middle of August when the plant has almost reached its maximum growth, but has not yet fully matured.

219

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IV

Tutt, M., Kikas, T., Olt, J. 2012.
Influence of different pretreatment methods on bioethanol production
from wheat straw.
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Influence of different pretreatment methods on bioethanol production from wheat straw

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Abstract. Article investigates the influence of different pretreatment methods on sugar conversion and bioethanol production. Different dilute acid and alkaline pretreatment methods are compared to determine the best pretreatment method to give the highest glucose and ethanol yields under the mild operating conditions. Wheat straw is used as a raw material as it is the most widely grown cereal in Europe. Dilute sulfuric acid, hydrochloric acid, nitric acid and potassium hydroxide solutions are used for pretreatment in combination with enzymatic hydrolysis. Results indicate that the highest cellulose-to-glucose conversion rate of 316.7 g kg^{-1} of biomass is achieved by the pretreatment with nitric acid. The lowest glucose concentration of 221.3 g kg^{-1} is achieved by hydrochloric acid. In the wheat straw samples pretreated with sulfuric acid and KOH, two different approaches are used. Solid phase of half the samples is rinsed with water before adding enzymes, and the rest of the samples are not. The rinsed samples pretreated with KOH solution give the highest ethanol yield of 104.3 g kg^{-1}, while the lowest ethanol yield is 67.7 g kg^{-1} from samples pretreated with HCl solution. Unrinsed samples and rinsed samples pretreated with sulfuric acid give an ethanol yield of 78.7 g kg^{-1} and 92.0 g kg^{-1}, respectively. These results indicate that rinsing the solid phase of the samples with distilled water before hydrolysis removes most of the inhibitory compounds formed during the pretreatment with dilute acid and increases fermentation efficiency by approximately 12%.

Key words: wheat straw, glucose, dilute acid pretreatment, cellulose

Introduction

Rising energy dependency on fossil fuels, increasing emissions of greenhouse gases and risks associated with the price fluctuations on the international energy markets has led to a move towards the research and production of alternative, renewable, efficient and cost-effective energy sources with lesser emissions (Dwivedi et al., 2009). Among many renewable energy alternatives for transportation fuels, four different energy sources are considered the most sustainable in the foreseeable future. These are biofuels, hydrogen, solar energy and syngas. At the moment, biofuels are considered the most favorable choice among these, because biofuels are renewable, biodegradable and cost-effective compared to using hydrogen or solar energy as transportation fuel (Nigam & Singh, 2011). Biofuels are classified as primary and secondary biofuels. The primary biofuels are natural and unprocessed biomass such as fuel-wood, wood chips and pellets. These are used by direct combustion for heating, cooking or power production. The secondary biofuels are produced by processing of biomass. For example, ethanol, biodiesel, methanol, etc. The secondary biofuels are

further divided into first, second and third generation biofuels on the basis of raw material and technology used for their production (Larson, 2008). Second–generation liquid biofuels are produced from lignocellulosic biomass, such as agricultural residues, grass and wood. It involves biological or thermochemical processing of the material to break the lignin structure and disrupt the crystalline structure of cellulose. The most widely produced second generation biofuel in the world is cellulosic ethanol (http://www.globalrfa.org/pr_021111.php).

Liquid biofuels are being researched mainly to replace conventional liquid fuels, such as diesel and petrol. The advantage of the second generation biofuels is the fact that it does not compete directly with the food market. It is possible to use entire above-ground biomass of a plant, thus enabling better efficiency and land use. Downside of the second generation biofuel production is the need for large investments and sophisticated processing equipment, compared to first generation (Stevens et al., 2004). In the future, the production of ethanol is expected to include both, traditional grain/sugar crops and lignocellulosic materials (Demirbas, 2011). Production of ethanol from lignocellulosic raw material and utilizing it as a substitute for petrol could help promote rural development, reduce greenhouse gases, and achieve independence from outside energy providers (Demirbas, 2005).

From different available raw materials wheat straw was chosen because it is the most widely grown cereal in Europe. For example, in 2004 the annual wheat straw production in Europe was approximately 132 million tons. Only a small portion of wheat straw is used for animal feed and bedding or for industrial use, and although the industrial use has been growing in the recent years, most of the wheat straw is still left on the fields or disposed of as waste (Sarkar, et al., 2012). Promoting the use of wheat straw as a raw material for bioethanol production could help increase the cellulosic ethanol production in Europe and reduce the quantity of biomass that goes to waste.

Several different pretreatment methods for wheat straw have been studied in the past, but no method has yet emerged as being efficient, but also simple and cost effective. Methods using moderate pretreatment conditions are cost effective, but usually have low sugar and ethanol yields. Pretreatment methods using high temperatures and harsh conditions have much better sugar and ethanol conversion yields, but they need expensive chemicals and equipment, thus making them economically not viable (Kim, et al., 2011).

Aim of this research was to investigate how different pretreatment methods with moderate conditions differ in hydrolysis and fermentation efficiencies. The influence of rinsing the solid phase of wheat straw samples on the sugar and ethanol conversion yields was also investigated.

Materials and methods

Biomass

Wheat straw was chosen as a raw material in this work, because it is the most widely grown cereal in Europe and much of the wheat straw is going to waste. Wheat is also grown in Estonia, and since straw does not compete directly with the food market, it makes wheat straw a good choice for bioethanol production.

270

Wheat straw samples were harvested in August, 2011, from the experimental fields of Estonian University of Life Sciences. Ash, hemicellulose, cellulose and lignin contents of straw samples were determined in the Laboratory of Plant Biochemistry of Estonian University of Life Sciences (see table 1).Standard methods of Association of Official Analytical Chemists (AOAC 973.18) and methods by company of Tecator (fibre determination using the Fibertec M&I systems) were used in the analysis. Samples were milled to a particle size of 1–3 mm and dried to a moisture content of less than 10%.

Table 1. Ash, hemicellulose, cellulose and lignin contents in dry mass of wheat straw samples

Sample	Ash %	Hemicellulose %	Cellulose %	Lignin %
Wheat straw	3.57	31.01	46.47	7.94

Pretreatment of a biomass

Cellulosic ethanol production is a complex process compared to first generation grain or sugarcane ethanol production. As the first step, it is necessary to break the lignin seal and hemicellulose sheathing over cellulose, and disrupt the crystalline structure of cellulose. Only then it is possible to degrade the cellulose in the biomass to sugar monomers. This disruption is achieved by the pretreatment process which is usually followed by enzymatic hydrolysis (Dwivedi et al., 2009; Kim et al., 2011).

Pretreatment with dilute acid

Pretreatment with dilute acid has been the most widely used method for pretreatment of the lignocellulosic material. This method uses cheap chemicals, mild operating conditions and is simple to perform. Downside of the dilute acid pretreatment method is a low conversion rate and formation of byproducts that are inhibitory for the following fermentation process. In the pretreatment with dilute acid, 0.5–1.5% sulfuric acid solution is added to the biomass to hydrolyse hemicellulose during 5–60 minutes at 130–200ºC. Higher temperatures require shorter time of pretreatment (Yang et al., 2009; Dien et al., 2006). Besides sulfuric acid, nitric acid also has shown good results in cellulose-to-sugars conversion yields, but nitric acids higher price makes it less cost effective.

Pretreatment with alkali

Pretreatment with alkali removes lignin and part of the hemicellulose, thus increasing the accessibility of enzymes to cellulose in later phases of hydrolysis. All of the cellulose and most of the hemicellulose is left in an insoluble polymeric form. This process uses alkali such as NaOH, KOH and $Ca(OH)_2$ and temperatures of 120–180°C. Pretreatment with alkali has been reported to give better ethanol yields than pretreatment with dilute acid. This is due to better fermentation efficiency, because formation of inhibitory byproducts is avoided. Downside of the method is a slightly lower sugar conversion rate. Pretreatment with alkali is best used for biomass with high lignin content (Gupta, 2008; Hamelinck et al., 2005; Mosier et al., 2005).

271

Enzymatic hydrolysis

The pretreatment is usually followed by enzymatic hydrolysis to convert the cellulose fibres and hemicellulose to fermentable sugars. Hydrolysis is carried out by different cellulase enzymes which are usually produced by lignocellulose degrading bacteria or fungi, for example *Trichoderma reesei*. The main factors that affect the hydrolysis rate of cellulose are accessibilty of cellulose fibers to enzymes, crystallinity of cellulose and hemicellulose, and lignin content (Sun & Cheng, 2002). Presence of lignin and hemicellulose makes the access of enzymes to cellulose fibres difficult. Therefore, the removal of lignin and hemicellulose as well as the increase of porosity during the pretreatment process increases the hydrolysis rate significantly (Dwivedi et al., 2009). At the same time, the presence of dissolved lignin can also inhibit the hydrolysis, so that not all of accessible cellulose is converted to sugars. Enzymatic hydrolysis can be carried out with total solid loadings up to 20%. If solid loading is higher than that, the constant stirring and equal distribution of enzymes in the mixture becomes difficult to achieve.

Analysis

Dilute sulfuric acid, hydrochloric acid, nitric acid and potassium hydroxide solution were used for pretreatment. The size of samples were 100 g of dried (moisture content <10%) and milled wheat straw to which 1,000 mL of 1% acid or alkaline solution was added. All samples were heated for $t = 60$ minutes at a temperature $T = 130 \pm 3°C$ and a pressure of $p = 3$ bar. As enzymes are inactivated when temperature $T > 70°C$ or $4 > pH > 7$, the sample was cooled to a temperature below 50°C and K_2CO_3 or HCl was added to neutralize the pH. Pretreatment was followed by enzymatic hydrolysis with the enzyme complex Accellerase 1,500. Enzyme mixture was added to the sample at a ratio of 0.3 mL per g of biomass. Hydrolysis lasted for $t = 24$ hours under constant stirring and at a temperature $T = 50°C$. After the hydrolysis process, glucose concentration in all of the samples was measured reflectometrically using RQflex 10 reflectometer and Reflectoquant glucose & fructose test. D–glucose and D–fructose are converted into D–glucose–6–phosphate. This is oxidized by NAD under the catalytic effect of glucose–6–phosphate dehydrogenase to gluconate–6–phosphate. In the presence of diaphorase, the NADH formed in the process reduces a tetrazolium salt to a blue formazan that is then determined reflectometrically.

In order to start the fermentation process, 2.5 g of dry yeast *Saccharomyces cerevisiae* was added to all of the samples. Fermentation process was carried out for 7 days under low oxygen conditions in 1,000 mL glass bottles, sealed with a fermentation tube. No glucose was detected in the samples after fermentation. Ethanol concentration was measured reflectometrically using RQflex 10 reflectometer and Reflectoquant alcohol test by Merck Inc. Under the catalytic effect of alcohol dehydrogenase, alcohol is oxidized by NAD to acetaldehyde. In the presence of an electron transmitter, the NADH formed in the process reduces a tetrazolium salt to a blue formazan that is determined reflectometrically.

At least 3 parallel samples were analyzed with each pretreatment method. Averaged results are used in figures and deviations are shown by vertical lines. Data was processed with programs Microsoft Excel and GraphPad Prism 5.

272

Results and discussion

The influence of different pretreatment methods on glucose and ethanol yields from wheat straw was investigated to determine the most efficient method for bioethanol production under moderate pretreatment conditions. Influence of washing the solid phase of wheat straw samples on the sugar and ethanol conversion yield was also investigated.

Results show that the highest cellulose to glucose conversion rate of 316.7 g kg^{-1} of biomass was achieved with the pretreatment by nitric acid (results shown in fig. 1). This indicates that nitric acid removes most of the hemicellulose from the sample and leaves the cellulose fibres easily accessible for enzymes. By far the lowest glucose yield of 221.3 g kg^{-1} was achieved by hydrochloric acid. Although the same acid concentrations were used, nitric acid pretreatment gave 30.1% higher glucose yield than the pretreatment with hydrochloric acid. This shows that 1% HCl acid solution is not strong enough to remove hemicellulose from the samples. Higher acid concentrations or longer pretreatment times could be used to overcome low glucose yield, but it would make the pretreatment with hydrochloric acid unfeasible compared to that with nitric acid or sulfuric acid.
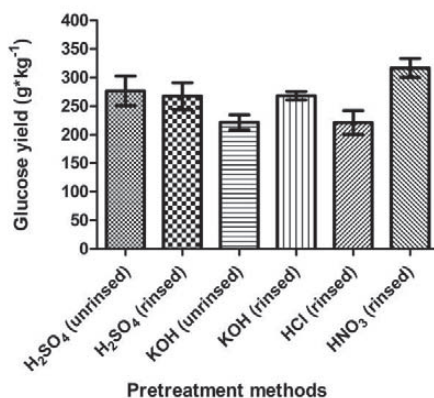
Fig. 1. The influence of different pretreatment methods on the glucose yield from wheat straw samples.

In wheat straw samples pretreated with sulfuric acid, two different approaches were used. Solid phase of half of the samples was rinsed with water before adding enzymes, and the rest of the samples were not. The results indicated that the unrinsed samples pretreated with sulfuric acid gave a glucose yield of 276.7 g kg^{-1}while samples that were rinsed before hydrolysis gave a glucose yield of 267.3 g kg^{-1}. Approximately 3.5% of cellulose is converted to sugars during the pretreatment with acid and is dissolved in the liquid phase. In case of pretreatment with diluted KOH, the unrinsed samples gave a glucose yield of 221.7 g kg^{-1}while samples that were rinsed before hydrolysis gave a glucose yield of 267.5 g kg^{-1}. This can be explained by the different

273

thickness of rinsed and unrinsed alkaline pretreated samples, presence of dissolved lignin and short hydrolysis time. Unrinsed samples were very thick and difficult to stir, and dissolved lignin in solution is a known inhibitor to enzyme activity (Berlin et al., 2006).

The rinsed samples pretreated with KOH gave the best ethanol yield of 104.3 g kg^{-1} results in fig. 2. On the other hand, wheat straw samples pretreated with HNO$_3$ gave an ethanol yield of only 95.0 g kg^{-1}regardless of the highest glucose yield of 316.7 g kg^{-1}. This can be explained by the formation of byproducts during acid pretreatment process which later inhibit fermentation process (Helle et al., 2003). Since these byproducts are not formed during alkaline pretreatment phase, the fermentation is more effective and more sugars are used for ethanol production rather than for the formation of organic acids and other unwanted byproducts.

Fig. 2. The influence of different pretreatment methods on the ethanol yield from wheat straw samples.

The effect of inhibitory compounds was seen in the wheat straw samples pretreated with sulfuric acid as well. The rinsed wheat straw samples pretreated with sulfuric acid gave approximately 14.5% higher ethanol yield than the samples that were not rinsed (ethanol yields of 92.0 and 78.7 g kg^{-1}, respectively). These results indicate that although washing removes 3.5% of sugars from a pretreated sample, it also removes a quantity of compounds that later inhibit the fermentation, thus resulting in a higher ethanol yield.

Results showed (table 2) that the samples pretreated with nitric acid (rinsed) had the best hydrolysis efficiency of 68.1%, but mediocre fermentation efficiency of 59.2%. In contrast, the samples pretreated with sulfuric acid (rinsed) had a hydrolysis efficiency of 57.5% and fermentation efficiency of 68.0%. This shows that nitric acid fractionates cellulose fibres and removes hemicellulose better than sulfuric acid, but byproducts of the pretreatment with nitric acid are more difficult to remove with rinsing and thus have a bigger negative impact on fermentation.

274

Table 2. Hydrolysis and fermentation efficiencies of different pretreatment methods

Pretreatment method	Glucose yield ($g\ kg^{-1}$)	Ethanol yield ($g\ kg^{-1}$)	Hydrolysis efficiency (%)	Fermentation efficiency (%)
H_2SO_4 (unrinsed)	276.7	78.7	59.5	56.1
H_2SO_4 (rinsed)	267.3	92.0	57.5	68.0
KOH (unrinsed)	221.7	77.0	47.7	68.3
KOH (rinsed)	268.2	104.3	57.7	76.3
HCl (rinsed)	221.3	67.7	47.6	59.9
HNO_3 (rinsed)	316.7	95.0	68.1	59.2

The highest fermentation efficiency of 76.3% was given by the samples pretreated with KOH (rinsed). This indicates that byproducts that impede fermentation are not formed during pretreatment with alkali. The downside of alkaline pretreatment method is its slightly lower hydrolysis efficiency compared to the dilute acid pretreatment methods. The alkaline pretreatment process removes lignin from samples, but leaves most of the hemicellulose intact which makes access of enzymes to cellulose fibres difficult. Dissolved lignin is also a known inhibitor of enzyme activity.

Conclusions

The aim of this research was to investigate the different pretreatment methods of wheat straw to find the most efficient and cost effective method using moderate pretreatment conditions. The influence of rinsing the solid phase of wheat straw samples after the pretreatment phase on the sugar and ethanol conversion yields was also investigated.

Samples pretreated with KOH (rinsed) gave the best ethanol yield of 104.3 $g\ kg^{-1}$ regardless of the glucose yields inferior to those of nitric acid and unrinsed sulfuric acid. The wheat straw samples pretreated with HNO_3 gave the highest glucose yield of 316.7 $g\ kg^{-1}$, but an ethanol yield of 95.0 $g\ kg^{-1}$ which was less than expected. This can be explained by the formation of byproducts during acid pretreatment process that later inhibit the fermentation process. Since byproducts that inhibit fermentation are not formed during alkaline pretreatment phase, the fermentation is much more effective and more sugars are used for ethanol production rather than for the formation of organic acids and other unwanted byproducts.
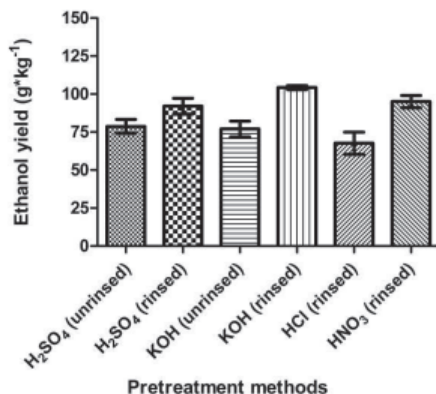
The samples pretreated with nitric acid (rinsed) had the best hydrolysis efficiency of 68.1%, but poor fermentation efficiency of 59.2%. It is in contrast to the results from the samples pretreated with sulfuric acid (rinsed) which had a hydrolysis efficiency of 57.5% and fermentation efficiency of 68.0%. This can be due to the fact that nitric acid fractionates cellulose fibres and removes hemicellulose better than sulfuric acid, but compounds that are formed during nitric acid pretreatment are more difficult to remove with washing and thus have a bigger negative impact on fermentation. Best fermentation efficiency of 76.3% was achieved by samples pretreated with KOH (rinsed). The downside of alkaline pretreatment method is its lower hydrolysis efficiency compared to sulfuric and nitric acid pretreatment methods.

In the light of these results we can conclude that from the point of ethanol production process under mild pretreatment conditions, the most effective method is KOH pretreatment process combined with rinsing the samples before the hydrolysis.

275

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276

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Nitrogen explosion pretreatment of lignocellulosic material for bioethanol production

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Abstract. A novel method for pretreatment of lignocellulosic material is investigated in this work, using floodplain meadow hay as a feedstock for bioethanol production. Pressurized nitrogen (N_2) pretreatment is combined with explosive decompression to achieve high glucose yields with simple technology and low energy input. Results show that N_2 explosion yields hydrolysis efficiencies up to 71.8%. The highest hydrolysis efficiency was achieved at a temperature of 210 °C with cellulose to glucose conversion rate of 195.1 g kg^{-1} of biomass.

Keywords: Cellulosic ethanol, biomass pretreatment, hydrolysis, floodplain meadow hay, steam explosion.

1. Introduction

Lignocellulosic raw materials represent the most abundant global resource for production of liquid biofuels at relatively low cost (Lin and Tanaka 2006; Talebnia et al. 2010). Problem with cellulosic ethanol production is that cellulose fibres are covered with hemicellulose and lignin layer that acts as a protective barrier and provides structural rigidity to cell walls, which has to be removed in order to convert cellulose to sugar monomers (Dwiwedi et al. 2009). This problem is overcome by using thermochemical or physical pretreatment methods.

Aim of this research was to investigate use of pressurized nitrogen as a pretreatment agent. Nitrogen molecules have similar size as water molecules, approximately 3.2 – 3.6 angstroms, and under pressure they dissolve in the water and can pass through plant cell walls. When the pressure is instantaneously released, gas volume expands explosively and dissolved nitrogen is released from the water, causing the cell structure to disrupt and expose cellulose fibres. Pretreatment phase was followed by enzymatic hydrolysis for the conversion of cellulose to sugar monomers. In order to assess suitability of the N_2 explosion pretreatment for sugar conversion and bioethanol production, the results from N_2 explosion were compared to those from steam explosion method.

2. Materials and methods

2.1 Biomass

Floodplain meadow hay was chosen as a sample feedstock, because there are over 20 000 hectares of floodplain meadows in Estonia which need regular maintenance and produce annually over 5 t/ha of biomass (Kukk and Sammul 2006; Heinsoo et al. 2010). Until now, there has been little use for this biomass. Floodplain meadow hay samples were harvested in July, 2012, from the floodplains of river Emajõgi. Samples were milled to a particle size of 1-3 mm and stored at room temperature. Dry matter content of samples was 90.4% (Tutt et al. 2014).

2.2 Pretreatment and analysis

In the N_2 explosion pretreatment sample of 100 g of pre-dried and milled hay was soaked in 500 g of distilled water and inserted into the reactor vessel. Reactor vessel was pressurized with N_2 gas to a pressure of $P = 30$ bar. Samples were heated in the reactor for $t = 15 - 120$ minutes to temperatures of $T = 70 - 210$ °C. After reaching the desired temperatures, reactor was cooled down to < 70 °C and pressure was released through a valve, see figure 1.
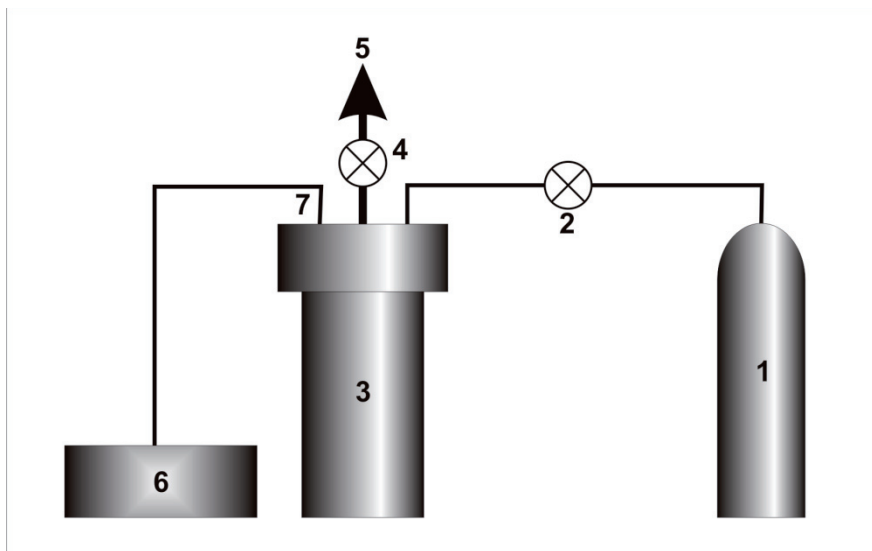
Fig. 1. Schematic of the nitrogen explosion pretreatment system (1-nitrogen tank, 2- pressure control valve, 3- reactor vessel with electric heating, 4- pressure release valve, 5- ventilation system, 6- controller unit, 7- thermocouple).

Steam explosion pretreatment was used in this work as a comparison method. Sample size was 900 g of pre-dried and milled hay, which was soaked in 900 g of distilled water. Steam explosion was carried out at temperatures $T = 150 - 200$°C and incubation times of $t = 10 - 30$ minutes. Pretreated material was then dried at temperature $T = 40$ °C to a dry matter content of 95%. Dry matter content was analysed with a moisture analyser Ohaus MB 45.

Pretreatment phase was followed by enzymatic hydrolysis with enzyme complex Accellerase 1500. Enzyme mixture was added to the sample at a

ratio of 0.3 mL per g of biomass. Hydrolysis of the pretreated material was carried out at 10% dry matter content in 1000 mL flasks. The samples were incubated for 24 h at 50°C in a shaking incubator at rotational speed of 250 rpm.

The fibre analysis (cellulose, hemicellulose and lignin) was performed according to the methods of Association of Official Analytical Chemists (AOAC 973.18) and methods by Tecator company. Saccharides, organic acids, furans in sample solutions were measured by high performance liquid chromatography (HPLC) (Eisenhuber et al. 2013).

Averaged results of at least three parallel measurements are used in figures and corresponding standard deviations are shown by vertical lines. All glucose yields are based on dry matter content.

3. Results and discussion

Sugar conversion yields from floodplain meadow hay using N_2 explosion pretreatment were compared to those from steam explosion pretreatment method.

Table 1. Cellulose, hemicellulose, lignin and dry matter contents of the untreated floodplain meadow hay (n=3).

	Cellulose (%)	Hemicellu lose (%)	Lignin (%)	Ash (%)	Dry matter (%)
Meadow Hay	27.19 ± 0.69	29.15 ± 0.48	24.16 ± 0.29	4.67 ± 0.18	90.43

The floodplain meadow hay sample was cut in the middle of July and thus, had fully matured. The biochemical composition of a plant cell wall changes during vegetation period (Kikas et al. 2014). This explains the results from fibre analysis which showed that sample had very high lignin content of 24.16%, but relatively low cellulose content of 27.19% (see Table 1) compared to some other lignocellulosic materials e.g. wheat straw, which had a cellulose content of 46.5% (Tutt et al. 2012).

The effect of two pretreatment methods at different temperatures on glucose yield is shown in Fig. 2.
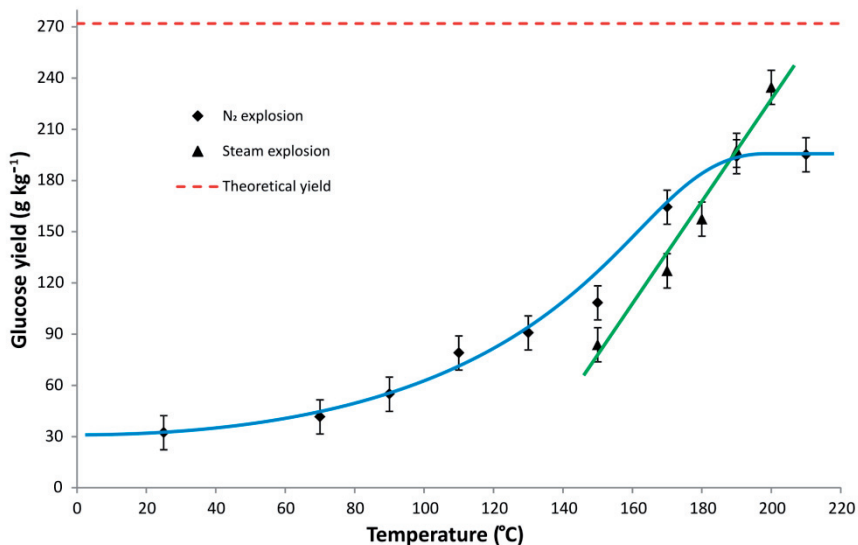
Fig. 2. Glucose yield dependence of N2 explosion and Steam explosion methods on the pretreatment temperature, where dashed line indicates the maximal theoretical glucose yield.

The glucose yield from biomass depends on the pretreatment method and temperature – N_2 explosion pretreatment when compared to steam explosion, was more efficient at lower temperatures while steam explosion enables to gain higher glucose yields at temperatures over 190°C. At that temperature the glucose yields of the two pretreatment methods are effectively the same, approximately 195 g kg^{-1}, as seen in Fig. 2. Even though the highest cellulose to glucose conversion yield of 195.1 g kg^{-1} of biomass with the N_2 explosion pretreatment was achieved at a temperature of 210°C, the change in the yield between 190°C and 210°C is negligible. The overall highest glucose yield of 234.6 g kg^{-1} of biomass was achieved with steam explosion pretreatment at 200°C. This is 16.8% better result than with N_2 explosion pretreatment and shows that steam explosion pretreatment is more efficient at temperatures over 190°C. The lowest glucose yield of 32.3 g kg^{-1} was achieved with N_2 explosion pretreatment at room temperature (25°C). This shows that explosive release of pressure alone, without thermal pretreatment, has little impact on plant cell wall structure. Although steam explosion

5

pretreatment enables to gain higher glucose yield than N_2 explosion pretreatment at temperatures over 190°C, at pretreatment temperatures between 150-180°C, explosive N_2 explosion pretreatment has higher glucose yields than steam explosion. This can be explained by lower pressure difference in steam explosion at temperatures 150-180°C. For example, absolute pressures of saturated steam at 150°C and 180°C were 4.9 bar and 10.1 bar, respectively. Higher pressure difference and higher temperatures gives higher impact factor to cell walls and better glucose yields. On the other hand, explosive release of pressure in N_2 pretreatment was always done at constant P = 30 bar.

Hydrolysis results show that efficiency of the N_2 explosion pretreatment and respective glucose yields increase with increasing temperature up to 190°C, see table 2. After that, increase in temperature has no further effect on the pretreatment efficiency.

In the case of steam explosion pretreatment, correlation between glucose yield and temperature change is best described by linear curve up to the theoretical value. This can be explained with the fact that pressure of the saturated steam in the pretreatment vessel changes with temperature (Westman 2008), but in N_2 explosion pretreatment the pressure was kept constant.

Table 2. Hydrolysis results at different pretreatment conditions (N- N_2 pretreatment at temperatures 25–210°C; SE- steam explosion pretreatment at temperatures 150–200°C).

Pretreatment method	Glucose yield g kg^{-1}	STD$_3$ g kg^{-1}	Hydrolysis efficiency %
N25	32.3	4.4	11.9
N70	41.5	2.6	15.3
N90	54.9	1.9	20.2
N110	79.0	1.4	29.1
N130	90.8	1.8	33.4
N150	108.3	12.4	39.8
N170	164.4	2.3	60.5
N190	193.9	10.3	71.3
N210	195.1	1.7	71.8
SE150	83.8	9.3	30.8

SE170	127.1	8.4	46.7
SE180	157.4	4.2	57.9
SE190	197.7	3.2	72.8
SE200	234.6	5.4	86.3

The highest hydrolysis efficiency of 86.3% was achieved by steam explosion at 200°C, while the highest hydrolysis efficiency of 71.8% with N_2 explosion pretreatment was achieved at 210°C. Glucose yields with N_2 explosion pretreatment did not rise significantly at temperatures over 190°C due to biomass charring. The latter could be associated with the combination of high temperature, high dry matter content inside the reactor and lack of mixing. In the future, there would be necessary to add mixing equipment to the reactor to avoid these problems. The greatest difference of efficiencies between N_2 explosion pretreatment and steam explosion was 13.8% at temperature of 170°C.

4. Conclusions

The highest cellulose to glucose conversion rate of 195.1 g kg^{-1} of biomass with the N_2 explosion was achieved at temperature of 210°C. Steam explosion is more efficient than N_2 explosion pretreatment at high temperatures, but less efficient at temperatures under 190°C. N_2 explosion pretreatment can be used as an effective pretreatment in bioethanol production even with a high-lignin biomass, such as floodplain meadow hay, and the sugar conversion yields from this method are comparable to steam explosion results.

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LIST OF CONFERENCE PRESENTATIONS

Oral presentations

Biosystems engineering, 10-11.05.2012, Tartu, Influence of different pretreatment methods on bioethanol production from wheat straw, Tutt, M., Kikas, T., Olt, J.

Engineering for rural development, 24-25.05.2012, Jelgava, Latvia, Comparison of different pretreatment methods on degradation of rye straw, Tutt, M., Kikas, T., Olt, J.

Biosystems engineering, 9-10.05.2013, Tartu, Influence of harvesting time on biochemical composition and glucose yield from hemp, Tutt, M., Kikas, T., Olt, J.

Biosystems engineering, 8-9.05.2014, Tartu, Using steam explosion pretreatment method for bioethanol production from floodplain meadow hay, Tutt, M., Pointner, M., Kahr, H., Kuttner, P., Kikas, T., Olt, J.

The Energy & Material Research Conference - EMR2015, 25-27.02.2015, Madrid, Spain, Formation of organic acids during bioethanol production from steam exploded floodplain meadow hay, Tutt, M., Kikas, T., Olt, J.

Biosystems engineering, 7-8.05.2015, Tartu, Factors affecting biochemical composition of lignocellulosic biomass and its effect on selection of pretreatment method and on bioethanol production potential, Tutt, M.

Poster presentations

Efficient and safe production processes in sustainable agriculture and forestry, XXXIV CIOSTA CIGR V Conference 2011, 29.06–1.07.2011. Vienna, Austria, Production of bioethanol from natural meadow biomass and herbaceous waste, Tutt, M., Olt, J.

Taastuvate energiaallikate uurimine ja kasutamine, TEUK XIII, 8.11.2011, Tartu, Erinevate taimeliikide võrdlus bioetanooli tootmiseks, Tutt, M., Olt, J.

International conference of agricultural engineering, CIGR-AgEng 2012, 8-12.07.2012, Valencia, Spain, Influence of different pretreatment methods on degradation of wheat straw, Tutt, M., Kikas, T., Olt, J.

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Taastuvate energiaallikate uurimine ja kasutamine, TEUK XVI, 13.11.2014, Tartu, Eeltöötlusmeetodi mõju bioetanooli saagisele, Tutt, M., Kikas, T., Olt, J.

VIIS VIIMAST KAITSMIST

REGINO KASK
THE INFLUENCE OF GROWTH CONDITIONS ON PHYSICO-MECHANICAL
PROPERTIES OF SCOTS PINE (Pinus sylvestris L.) WOOD IN ESTONIA
KASVUTINGIMUSTE MÕJU HARILIKU MÄNNI (Pinus sylvestris L.) PUIDU
FÜÜSIKALIS-MEHAANILISTELE OMADUSTELE EESTIS
Dots. Emer. **Jaak Pikk**
15. juuni

TÕNU TÕNUTARE
POSSIBILITIES TO AFFECT ANTIOXIDANT PROPERTIES OF STRAWBERRIES
AND SOME METHODICAL ASPECTS IN THEIR DETERMINATION
AEDMAASIKA VILJADE ANTIOKSÜDATIIVSETE OMADUSTE MÕJUTAMISE
VÕIMALUSTEST JA METOODILISTEST ASPEKTIDEST NENDE MÄÄRAMISEL
Dotsent **Ulvi Moor**
15. juuni

PRIIT PÕLLUMÄE
ASSESSMENT OF PRIVATE FOREST OWNERS' COOPERATION IN ESTONIA
EESTI ERAMETSAOMANIKE KOOSTÖÖ ANALÜÜS
Dotsent **Henn Korjus**
21. august

PRIIT-KALEV PARTS
SUSTAINABLE COMMUNITY MANAGEMENT IN ESTONIA: REFLECTIONS ON
HERITAGE PROJECTS ON KIHNU ISLAND, IN VILJANDI COUNTY,
AND IN VARIOUS PROTECTED AREAS
KESTLIK KOGUKONNAKORRALDUS: KULTUURIPÄRANDIGA SEOSTUVATE
ARENDUSTEGEVUSTE PEEGELDUSI KIHNU SAARELT, VILJANDI
Professor **Kalev Sepp**
21.august

BERT HOLM
CULTIVATION OF WILLOWS (SALIX SP.) USING RESIDUES
OF THE WASTEWATER PURIFICATION PROCESS
AND BIOGAS PRODUCTION AS FERTILISERS
PAJUDE (SALIX SP.) KASVATAMINE KASUTADES VÄETISTENA
REOVEEPUHASTUSPROTSESSI- JA BIOGAASI TOOTMISE JÄÄKE
Vanemteadur **Katrin Heinsoo**
21. august