

**Dissertationes Forestales 202**

The use of lignocellulosic biomass for fermentative  
butanol production in biorefining processes

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Academic dissertation

To be presented, with the permission of the Faculty of Science and Forestry of the University of Eastern Finland, for public examination in the auditorium Futura 100 of the University of Eastern Finland, Yliopistonkatu 7, Joensuu, on 9<sup>th</sup> October 2015, at 12 o'clock noon.

*Title of dissertation:* The use of lignocellulosic biomass for fermentative butanol production in biorefining processes

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*Dissertationes Forestales* 202

<http://dx.doi.org/10.14214/df.202>

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ISSN 1795-7389 (online)

ISBN 978-951-651-493-5 (pdf)

ISSN 2323-9220 (print)

ISBN 978-951-651-494-2 (paperback)

*Publishers:*

Finnish Society of Forest Science

Natural Resources Institute Finland

Faculty of Agriculture and Forestry at the University of Helsinki

School of Forest Sciences at the University of Eastern Finland

*Editorial Office:*

The Finnish Society of Forest Science

P.O. Box 18, FI-01301 Vantaa, Finland

<http://www.metla.fi/dissertationes>

**Yang, M. 2015.** The use of lignocellulosic biomass for fermentative butanol production in biorefining processes. *Dissertationes Forestales* 202. 49 p. Available at: <http://dx.doi.org/10.14214/df.202>

## ABSTRACT

The aim of this study was to efficiently use barley straw as a lignocellulosic feedstock for biobutanol production. Dilute sulfuric acid pretreatment was employed to solubilize hemicellulose in barley straw from cellulosic residues. The pretreated hydrolysate was co-fermented with starch-based biomass in acetone–butanol–ethanol (ABE) fermentation. There were two co-fermentation processes: I) Barley straw was mixed with barley grain, and the mixture was pretreated with dilute acid pretreatment. The sugars mainly released from hemicellulose and starch into the pretreated hydrolysate of the mixture were fermented for biobutanol production; II) Pretreated barley straw hydrolysate and gelatinized barley grain slurry was mixed and fermented. The pretreated solid residues containing mostly the cellulosic biomass was used for efficient enzymatic hydrolysis by synergistic cooperation of cellulases with xylanase and surfactants to produce fermentable sugars, and followed by ABE fermentation. Furthermore, the use of fresh barley silage for biobutanol production as an example of the co-fermentation process was investigated.

By pretreatment of a mixture of barley straw and grain (process I), optimal fermentable sugar yields were obtained under the pretreatment condition with 1.5% sulfuric acid in 60 min. However, the pretreatment with 1.0% sulfuric acid resulted in better ABE fermentability of the hydrolysate mixture (M1.0) than that with 1.5% sulfuric acid. The fermentation of M1.0 produced 11.3 g/L ABE, but only 19% of pentoses were consumed. In process II, fermentation of the mixture of pretreated straw hydrolysate and gelatinized grain slurry produced more ABE (13.5 g/L) than that in process I, and 95% of pentoses were utilized in the hemicellulosic hydrolysate pretreated with more severe condition (1.5% sulfuric acid). The use of pretreated hydrolysate defined as pretreatment liquor from green and yellowish barley silage supplemented with gelatinized barley grain slurry showed feasibility for ABE fermentation, and 9.0 g/L and 10.9 g/L total ABE was produced, respectively. The combined application of xylanase and PEG 4000 in the hydrolysis of pretreated solid residues by cellulase increased the glucose and xylose yields, which were considerably higher than that obtained with the application of either one of them. The enhanced sugar production increased ABE yield from 93.8 to 135.0 g/kg pretreated straw.

The results suggest that it is feasible to ferment the hemicellulosic biomass with starch-based biomass, and improve the sugar production from cellulosic biomass in straw by combined application of xylanase and surfactants in enzymatic hydrolysis for biobutanol production. For the efficient utilization of hemicellulosic biomass, the process II is more favorable, particularly for sustainable biofuel production from variety of lignocellulosic feedstocks. The enzymatic hydrolysate obtained by additive xylanase and surfactants showed good fermentability in biobutanol production, and the efficiency of straw utilization was apparently increased. Moreover, the pretreatment liquor of fresh barley silage was efficiently used for butanol fermentation with the co-fermentation processes, indicating the feasibility of utilization of green field biomass preserving by “silage” technique in biorefining processes.

**Keywords:** Biofuel, acetone–butanol–ethanol, barley straw, green field biomass, pretreatment, enzymatic hydrolysis, sugars

## ACKNOWLEDGEMENTS

My greatest appreciation goes to my supervisor, Prof. Ari Pappinen for leading me to his research group in University of Eastern Finland. Without his guidance and support, this thesis would not have been completed successfully. I would like to express my special thanks to my supervisor, Markku Keinänen, for his advising in writing articles and technical support on Gas chromatography-mass spectrometry (GC-MS) analysis. Next, to Suvi Kuittinen, I am sincerely grateful for her kind guidance on my studies, laboratory work, and comments on the articles, which helped them to be published successfully. I would like to give special acknowledgement to Prof. Junhua Zhang for the suggestions on the experiment conduction and comments on the articles. Also, thanks go to Prof. Jouko Vepsäläinen for his kind technical support on Nuclear magnetic resonance (NMR) analysis and comments on the articles. Thanks are extended to Prof. Pasi Soininen for the help with the NMR measurements. I also appreciate Dr. Jinnan Gong for the comments on Article I, and Dr. Markku Huttunen for the comments on the thesis. Warm thanks go to the pre-examiners Docent Ossi Turunen and Prof. Juha Tanskanen for reviewing and comments.

The work was part of the project “Renewable transportation fuels in North Karelia-development of expertise” financed by European Structural Funds, led by Prof. Ari Pappinen. The work was conducted at school of Forest Sciences, University of Eastern Finland. The work was also financed by Doctoral programme in Forests and Bioresources in University of Eastern Finland. I also obtained research grant from the Niemi Foundation, and Finnish Cultural Foundation. Thanks a lot for all the financial supports.

Words cannot express how grateful I am to my wife, Aifang Wang and my son, Zixuan Yang. Without their love and support, I would not complete the study and enjoy the life in Finland. The appreciation also goes to my parents, brother, and sister for their continuous support. Finally, I wish to thank my friend Henri Vanhanen for the encouragement on my study, and kind support for us in living in Finland. I also give my thanks to Riikka Linnakoski, Yasuhito Sugano and Kamrul Hassan, for sharing their experiences on doctoral studies with me. Thanks also go to my other Chinese, Finnish and international friends for the splendid moments we spent together.

Ming Yang

Joensuu, September 2015

## LIST OF ORIGINAL ARTICLES

The thesis is based on the following articles, which are referred to in the text by the Roman numerals I-IV. Articles I-IV are reproduced with the kind permission of publishers.

- I Yang M., Kuittinen S., Zhang J., Keinänen M., Pappinen A. (2013). Effect of dilute acid pretreatment on the conversion of barley straw with grains to fermentable sugars. *Bioresource Technology* 146: 444–450.  
doi: 10.1016/j.biortech.2013.07.107
- II Yang M., Kuittinen S., Zhang J., Vepsäläinen J., Keinänen M., Pappinen A. (2015). Co-fermentation of hemicelluloses and starch from barley straw and grain for efficient pentoses utilization in acetone-butanol-ethanol production. *Bioresource Technology* 179: 128–135.  
doi: 10.1016/j.biortech.2014.12.005
- III Yang M., Zhang J., Kuittinen S., Vepsäläinen J., Soininen P., Keinänen M., Pappinen A. (2015). Enhanced production of reducing sugars from pretreated barley straw by additive xylanase and surfactants in enzymatic hydrolysis for acetone-butanol-ethanol (ABE) fermentation. *Bioresource Technology* 189: 131–137.  
doi: 10.1016/j.biortech.2015.04.008
- IV Yang M., Kuittinen S., Keinänen M., Vepsäläinen J., Romar H., Tynjälä P., Lassi U., Pappinen A. (2014). The use of (green field) biomass pretreatment liquor for fermentative butanol production and the catalytic oxidation of biobutanol. *Chemical engineering research and design* 92: 1531–1538.  
doi: 10.1016/j.cherd.2014.01.015

### The author's contribution

Ming Yang was responsible for running the experiments, data analysis, and wrote the manuscripts. The co-authors all contributed to the articles through comments for improvement on publication. The research ideas were developed by Ari Pappinen, Suvi Kuittinen and Ming Yang. Markku Keinänen advised in writing the manuscripts and GC-MS analysis. Junhua Zhang advised in designing the experiments. Jouko Vepsäläinen and Pasi Soininen contributed on the samples analysis by NMR. The catalytic oxidation of butanol in Article IV, and the writing on this part was done by co-authors Ulla Lassi, Henrik Romar and Pekka Tynjälä.

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## LIST OF ABBREVIATIONS

ABE	Acetone-butanol-ethanol
GC-MS	Gas chromatography-mass spectrometry
NMR	Nuclear magnetic resonance
IEA	International Energy Agency
DP	Degree of polymerization
LCCs	Lignin-carbohydrate complexes
INL	Idaho National Laboratory
DM	Dry matter
NREL	National Renewable Energy Laboratory
DNS	3,5-dinitrosalicylic acid
DDGS	Distillers dried grains and solubles
AFEX	Ammonia fiber explosion
HMF	5-hydroxymethylfurfural
PEG	Polyethylene glycol
SSF	Simultaneous saccharification and fermentation
CS	Combined severity
PHs	Pretreated hydrolysates
SRFs	Solid residual fractions
CEL	Celluclast 1.5 L and Novozyme 188
CXP	CEL + xylanase + PEG 4000



## 1. INTRODUCTION

Petroleum and its derivatives are extensively used in modern society. The issues of global warming and climate change have raised the concerns of biorefining processes, particularly biofuels production as substitutes for petroleum-derived transportation fuels. Biofuels are produced from renewable bioresources, and therefore have a strategic advantage to supplement conventional energy source, promoting the sustainable development of energy supply system. In 2013, more than 115 billion litres biofuels were produced in the world. This amount contributed around 3.5% of global consumption of road transport fuel. It is estimated that by 2050, biofuels could provide 27% of total transport fuel, avoiding around 2.1 gigatonnes (Gt) of CO<sub>2</sub> emissions per year when produced sustainably (IEA 2014).

The most common liquid biofuel worldwide is ethanol made by fermenting sugars from biomass. It can be blended up to 15% with gasoline used in most existing car engines. Biodiesel is another transportable fuel which is nontoxic, biodegradable and renewable used for vehicles in its pure form. Currently, the demand of biofuels is mostly met by ethanol and biodiesel. However, bioethanol has low energy density, and its hygroscopicity poses a problem for storage and distribution (Bongaerts and Abreu 2012). Limited availability of fat and oil resources is one of the challenges for biodiesel production (Ma and Hanna 1999). There is a need for advanced fuels with superior technical properties and with first class sustainability credentials. Butanol, a competitive renewable biofuel, is regarded as an ideal biofuel in the future with many advantages over bioethanol: 1) it has a higher heating value and lower volatility due to the increased carbon atom number; 2) the lower heat of vaporization of butanol causes less ignition problems; 3) low vapor pressure point and high flash point of butanol makes it much safer for use in high temperatures; 4) it is easier for distribution because it is less corrosive; 5) it can be blended in gasoline at higher concentration without modification of vehicles (Jin et al. 2011).

During the last two decades, the research and industrial production of biofuels has progressed, but process development is still needed due to problems with, for example, high production costs, low productivity and lack of commercial bacteria owning optimal characteristics. In recent years, the production of biofuels such as bioethanol from corn or wheat has faced heavy criticism regarding their sustainability. Rising food price and actual effectiveness to reduce global carbon emissions have led to controversies about their use in biofuel production. However, biofuels are associated with considerable benefits for the reduction of dependency on crude oil and diversity in energy supply. Using also the lignocellulose, part of the crop biomass as feedstock could avoid the competition with food production. Potentially, it could push the agricultural economic development in the developing regions. The sustainability of transportable biofuels production should be assessed throughout the life cycle.

## 2. REVIEW OF LITERATURE

### 2.1 Lignocellulosic raw materials

Lignocellulosic biomasses such as agricultural and forest residues, dedicated energy crops, and industrial and municipal wastes are the most abundant feedstocks, and hold tremendous

potential for large scale biofuels production. However, the effective utilization of lignocellulose is not always practical due to the recalcitrant structure of lignocellulose to hydrolysis. Lignocelluloses are mainly composed of cellulose, hemicellulose and lignin, in addition to a small amount of pectin, starch, minerals (ash) and extractives. Various lignocellulosic biomasses and different contents of the three major components shown in Table 1 affect the efficacy of the conversion technology.

Cellulose is the main structural constituent of plant cell wall, it is a polysaccharide of thousands of D-glucose units linked via  $\beta$ -(1-4) glycosidic bonds and with a degree of polymerization (DP) of up to 10 000 or higher (Jørgensen et al. 2007). The second most abundant plant polysaccharide is hemicellulose. It is composed of pentoses (xylose, arabinose), hexoses (mannose, glucose and galactose) and acylated sugars. The degree of polymerization of hemicellulose is below 200. Lignin is a complex network containing cross-linked polymers of three phenolic monomers (p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol). Lignin is a non-polysaccharide in lignocellulose, and embeds the cellulose to protect against microbial and chemical degradation. It is generally acknowledged that feedstocks with larger quantities of cellulose and hemicellulose are favored with the current conversion technology. The polysaccharides in lignocelluloses naturally resist chemical, physical and enzymatic degradation, and lignin blocks access of enzymes for hydrolysis. The digestibility of lignocellulosic biomass is directly determined by the structure and content of lignin, content of hemicelluloses, lignin-carbohydrate complexes (LCCs), cellulose crystallinity and DP, pore volume, and specific area of cellulose (Hu and Ragauskas 2012). Efficient pretreatment of lignocellulosic biomass is necessarily employed to enhance the digestibility of lignocellulose.

**Table 1.** Three main components in lignocellulosic biomass<sup>a</sup>.

Lignocellulosic materials		Cellulose (%)	Hemicellulose (%)	Lignin (%)
Forest residues	Hardwood	40-55	24-40	18-25
	Softwood	45-50	25-35	25-35
	Leaves	15-20	80-85	0
Agricultural residues	Corn stover	31-35	19-44	13-21
	Wheat straw	30	50	15
	Corn cobs	45	35	15
	Nut shells	25-30	25-30	30-40
	Cotton seed hairs	80-95	5-20	0
	Grasses	25-40	35-50	10-30
Energy crops	Herbaceous energy crops	45	30	15
	Switchgrass	45	31.4	12
	Miscanthus	43	24	19
	Coastal Bermuda grass	25	35.7	6.4
	Paper	85-99	0	0-15
Industrial and municipal wastes	Newspaper	40-55	25-40	18-30
	Waste papers from chemical pulps	60-70	10-20	5-10
	Primary wastewater solids	8-15	Not available	24-29
	Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
	Swine waste	6	28	Not available

<sup>a</sup> Source: Reshamwala et al. 1995, Cheung and Anderson 1997, Boopathy 1998, Dewes and Hünsche 1998, Sun and Chen 2002.

In addition to the conversion efficiency, the use of lignocellulosic biomass for biofuel production may not be cost-effective due to the complex feedstock supply chains that include biomass harvest and collection, storage, preprocessing, and transportation (Banerjee et al. 2010). Research of feedstock logistics is needed before the potential of lignocellulose can be fully utilized. An advanced feedstock supply system to reduce the costs could include innovative ways in logistics, and the improved technologies to increase the operation efficiency and biomass quality (INL 2014).

## 2.2 Pretreatment

Pretreatment disrupts the heterogeneous structure of lignocellulose, removes hemicelluloses and/or lignin, increases the surface area and porosity of biomass, and reduces the crystallinity of cellulose, thus increasing the accessibility of cellulose to enzymes (Wyman et al. 2005, Mosier et al. 2005). It has been proved that removal of lignin and to a less extent hemicelluloses is effective in increasing the cellulose hydrolysis (Chang et al. 2000, Kim et al. 2003, Pan et al. 2005). In a recent study, a strong correlation of enzymatic conversion yield with the average pore size of the starting material (cellulose) was found, but there was no significant correlation between the conversion yield with the surface area, lateral fibril dimensions and degree of crystallinity of substrate (Peciulyte et al. 2015). Although pretreatment is necessary for efficient conversion of lignocelluloses to fermentable sugars, nearly 18-20% of the total projected cost is attributed to pretreatment in bioethanol production (Yang et al. 2007). In general, pretreatments should have a low capital and operational cost, a wide effective range for different materials, a maximum enzymatic convertibility, and a low amount of sugar degradation components in the products. Many pretreatment technologies including physical, chemical, physico-chemical and biological methods have been studied, and the pretreatment conditions, advantages and disadvantages are summarized in Table 2.

Among these technologies, dilute acid pretreatment, alkaline pretreatment and hydrothermal pretreatment (e.g. steam explosion and liquid hot water pretreatment) are the most common technologies being developed. However, combinatorial pretreatment strategies are usually preferred in designing leading pretreatment technologies, since they are generally more effective in enhancing the biomass digestibility. Steam explosion and ammonia fiber explosion (AFEX) are good examples of physico-chemical technologies for pretreatment of agricultural residues such as corn stover, wheat straw, rich straw, barley straw and sugarcane bagasses. A combined dilute acid and steam explosion pretreatment of rice straw resulted in a higher xylose yield, a lower level of inhibitors in the hydrolysate and a greater degree of enzymatic hydrolysis (Chen et al. 2011). Many other combined pretreatments have also been reported, for example, microwave-assisted dilute sulfuric acid pretreatment of rape straw (Lu et al. 2011), Plasma-Assisted (Ozone generated in a plasma at atmospheric pressure and room temperature) pretreatment of wheat Straw (Schultz-Jensen et al. 2011), alkaline peroxide pretreatment of corn stover (Banerjee et al. 2011), crude glycerol and ionic liquids pretreatment of wheat straw and water hyacinth (Guragain et al. 2011), and fungal pretreatment combined with a mild alkali treatment of wheat straw (Salvachúa et al. 2011).

**Table 2.** Various pretreatment methods and their advantages and disadvantages<sup>a</sup>.

Pretreatment Methods		Common conditions	Advantages	Disadvantages
Physical	Mechanical comminution	Chipping to 10-30 mm, milling to 0.2-2 mm	Increase surface area, decrease DP and cellulose crystallinity	Energy-intensive
	Steam explosion	Saturated steam at 160-260°C 1-2 kg of ammonia/kg biomass, at 60-90°C, 10-60 min	Hemicellulose degradation and lignin transformation	Inhibitors, not really effective with softwood
	Ammonia fiber explosion		Removes lignin and hemicellulose to an extent, no inhibitors	Not efficient for biomass with high lignin content
Physico-chemical	CO <sub>2</sub> explosion	Supercritical CO <sub>2</sub> under pressure	Cost-effective, no inhibitors, low temperature and high solid capacity	Limited equipment for large scale
	Liquid hot water pretreatment	Temperature 180-190°C and dry matter 1-8%	Lower temperature, minimum degradation products, low cost	Energy cost of downstream process, not successful with softwood
	Ionic liquid pretreatment	With imidazolium salts	Environmental friendly, Removes lignin	High cost, process under investigation
Chemical	Ozonolysis	With ozone, low temperature and pressure	Removes lignin, no toxic residues,	Cost of ozone, Inefficient
	Acid hydrolysis	< 4 wt.% sulfuric acid or HCl	Hydrolyzes hemicellulose, alters lignin structure	High cost, equipment corrosion, toxic substances
	Alkaline hydrolysis	Low temperature, long time, high concentration of base	Disrupts lignin structure, breaks the linkage between lignin and carbohydrates	Long time, irrecoverable salts incorporated into biomass
	Oxidative delignification	Oxidizing agent	Lignin degradation	High cost of agents, inhibitors
	Organosolv process	Organic solvents or mixture with inorganic acid catalysts	Hydrolyzes lignin and hemicelluloses	High cost of solvent evaporation, condensation, and recycling
Biological	Biological	Fungi	Degrades lignin and hemicelluloses, low energy requirements	Low rate of hydrolysis

<sup>a</sup> Source: Sun and Chen 2002, Mosier et al. 2005, Kumar et al. 2009, Alvira et al. 2010, Agbor et al. 2011, Limayem and Ricke 2012.

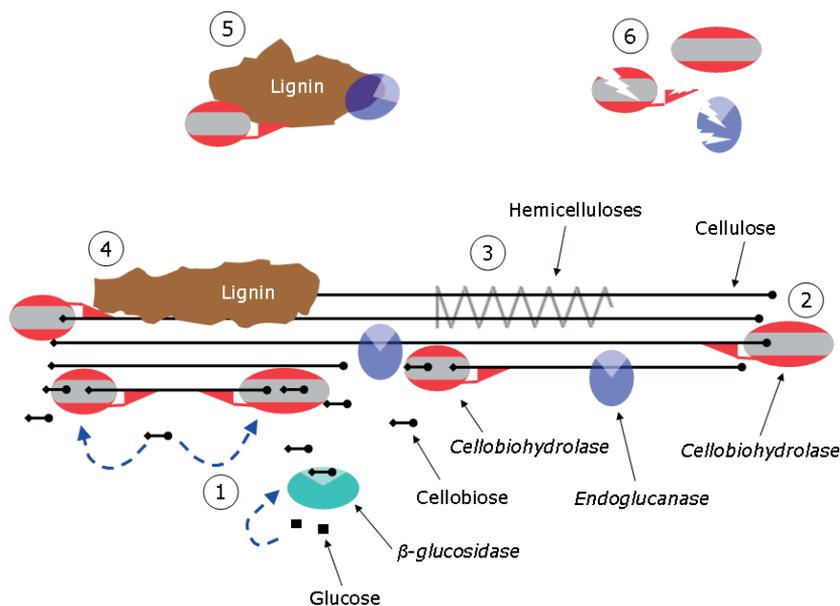
In some commonly used pretreatments, such as steam explosion and dilute acid pretreatment, certain chemicals including 5-hydroxymethylfurfural (HMF), furfural, weak organic acids and phenolic compounds are formed as sugar and lignin degradation products which have been shown to inhibit enzymatic hydrolysis and biofuels fermentation (Cantarella et al. 2004, Ezeji et al. 2007a). To avoid the problems caused by these inhibitors, the process of detoxification is sometimes required. Detoxification can be performed with different methods, such as the usage of ion exchange (Horváth et al. 2004), laccase (Jurado et al. 2009), activated charcoal (Mussatto and Roberto 2004) and wood ash treatment (Miyafuji et al. 2003), alkali treatment (Persson et al. 2002). However, a problem associated with detoxification is that the treatment may also affect the sugars, which would lead to reduced ethanol yield (Nilvebrant et al. 2003). In addition, special design of the fermentation process, selection of high resistant microorganisms could also be performed to reduce the negative effect of inhibitors.

### 2.3 Enzymatic hydrolysis

Enzymatic hydrolysis of pretreated lignocellulosic materials represents the most effective method to produce simple sugars for biofuels fermentation. Compared to acid hydrolysis, enzymatic hydrolysis is more efficient with milder operating conditions, results in better sugar yields, and uses less chemical input (Banerjee et al. 2010, Yang et al. 2011). Enzymatic hydrolysis of pretreated lignocellulosic materials involves enzymatic reactions that convert cellulose into glucose and hemicellulose into pentoses (xylose and arabinose) and hexoses (glucose, galactose, and mannose).

Cellulose hydrolysis is catalyzed by three different classes of enzymes: (1) Endoglucanase (EG, endo-1,4-D-glucanohydrolase, EC 3.2.1.4.) which hydrolyzes internal  $\beta$ -1,4-D-glucosidic linkages randomly in the cellulose chain; (2) exoglucanase or cellobiohydrolase (CBH, 1,4- $\beta$ -D-glucan cellobiohydrolase, EC 3.2.1.91.) which further removes cellobiose units from the free chain-ends; and (3)  $\beta$ -glucosidase (EC 3.2.1.21) which hydrolyzes cellobiose to glucose and cleaves off glucose units from cellooligosaccharides (Sun and Chen 2002, Jørgensen et al. 2007). These three groups of enzymes function synergistically for cellulose hydrolysis by creating new accessible sites for each other, and partly preventing product inhibition.

Hemicelluloses are usually removed in pretreatment process, but sometimes they are left in the materials and need efficient hydrolysis by a series of hemicellulases, which participate in the hydrolysis of xylan and glucomannan. Endo-1,4- $\beta$ -D-xylanases (EC 3.2.1.8) hydrolyze xylan chain to oligomers; 1,4- $\beta$ -D-xylosidases (EC 3.2.1.37) attack xylooligosaccharides from the non-reducing end and liberate xylose (Jørgensen et al. 2007). Endo-1,4- $\beta$ -D-mannanases (EC 3.2.1.78) hydrolyze internal bonds in glucomannan chain, and the formed oligosaccharides are further hydrolyzed by 1,4- $\beta$ -D-mannosidases (EC 3.2.1.25) and  $\beta$ -glucosidase (EC 3.2.1.21) (Jørgensen et al. 2007). The side groups of xylan and glucomannan hinder the action of endo-1,4- $\beta$ -D-xylanases and endo-1,4- $\beta$ -D-mannanases. A number of enzymes such as  $\alpha$ -D-galactosidases (EC 3.2.1.22),  $\alpha$ -L-arabinofuranosidases (EC 3.2.1.55),  $\alpha$ -glucuronidases (EC 3.2.1.139), acetyl xylan esterases (EC 3.1.1.72) and feruloyl and p-coumaric acid esterases (EC 3.1.1.73) can remove the side groups (Beg et al. 2001, Shallom and Shoham 2003).



**Figure 1.** The limiting factors for efficient hydrolysis of cellulose ①: Glucose and cellobiose inhibition of  $\beta$ -glucosidases and cellobiohydrolases by glucose and cellobiose, respectively; ②: Unproductive binding of cellobiohydrolases onto cellulose chain; ③ and ④: Hemicelluloses and lignin blocks the access of cellulase to cellulose surface; ⑤: Unspecific adsorption of enzymes into lignin surfaces; ⑥: Denaturation or inactivation of enzymes due to their low thermostability, mechanical shear or proteolytic activity (adapted from Jørgensen et al. 2007).

Enzymatic hydrolysis of lignocellulose into fermentable sugars is one of the most expensive steps in biofuel production depending on the cost of the enzymes. Enzymes should be efficiently used in the hydrolysis, however, there are number of obstacles which affect the enzyme performance (Fig. 1). During recent years, the technologies to improve the enzymatic hydrolysis of lignocellulosic materials have been extensively developed. The employment of suitable pretreatment method could remove the physical barrier of cellulases accessing to cellulose formed by hemicellulose and lignin. Alkaline peroxide treatment removed 80% of the lignin in steam exploded softwood that improved the enzymatic conversion and lowered the enzyme loading significantly (Yang et al. 2002). Simultaneous saccharification and fermentation (SSF) process reduces the inhibition of end products of hydrolysis, and requires relatively low amounts of enzyme (Alfani et al. 2000, Wingren et al. 2003). Certain additives, in particular surfactants such as Tween and ethylene oxide polymers like polyethylene glycol (PEG) have shown the potential to increase hydrolysis efficiency by affecting the hydrophobic interaction between lignin surfaces and enzymes (Eriksson et al. 2002, Börjesson et al. 2007). As reported previously, surfactant addition increased ethanol yield by 8% and reduced cellulase loading by as much as 50% (Alkasrawi et al. 2003). The synergistic cooperation between enzymes affects the biomass conversion, for example, the addition of xylanase improves the performance of cellulases and increases cellulose conversion of pretreated hardwoods and softwoods (Berlin et al. 2005, Berlin et al. 2006, Van et al. 2012). In addition, recycling of the enzymes is an attractive way of reducing enzymes cost and

improving the hydrolysis efficiency (Gregg et al. 1998). High-temperature enzymatic hydrolysis improves reaction rates and mass transfer, and lowers viscosity. The prehydrolysis (2h) of steam pretreated spruce with *Pyrococcus horikoshii* endoglucanase at 100°C improved the cellulase hydrolysis by 20-30%; and with xylanase and mannanase prehydrolysis at 80°C (2 h) hydrolysis yield increased by 20-33%, while all three enzymes together improved the final enzymatic yield by 50-70% (Hämäläinen et al. 2015).

## 2.4 Biobutanol production from lignocellulosic biomass

Biobutanol can be produced through traditional acetone–butanol–ethanol (ABE) fermentation by *Clostridium spp.* using a variety of substrates including monosaccharides (hexose and pentose) and polysaccharides (starch) (Madiah et al. 2001, Ezeji and Blaschek 2008, Sun and Liu 2012, Survase et al. 2012). At present, sugar-based feedstocks (sugarcane and sugar beet molasses), and starch-based feedstocks (corn and wheat) are mainly used for butanol production by *Clostridium* fermentation. *Clostridium* possesses strong amylase activities to hydrolyze starch for ABE fermentation. However, the increasing demand for sugar and starch-based substrates for biofuel production has increased the prices of these feedstocks. Importantly, these crops have recently raised the food vs energy debates, and they are not sustainable for large scale butanol production. The possible solution for obtaining enough fermentable carbon substrates without getting into the competition with food supplies is the efficient utilization of plentiful lignocellulosic biomass available on earth.

Treatment of lignocellulosic biomasses results in a mixture of sugars, which contains pentoses, hexoses and disaccharides. It was also reported that pentoses released from hemicellulose are usually wasted in most cellulosic ethanol pilot and demonstration plants due to low fermentability by the most common industrial microbial strains (Girio et al. 2012). From an economical point of view, efficient utilization of all the extracted sugars in lignocelluloses for ABE fermentation seems to be a good strategy for sustainable biofuels production (Lee et al. 2008, Jurgens et al. 2012). However, in addition to the efficiency of lignocellulose utilization, the butanol production is also limited by various factors, such as butanol toxicity to *Clostridium spp.*, low cell density in the fermentation, and the high cost of butanol recovery due to the formation of by products and low butanol concentration. ABE production from lignocellulosic feedstocks have been reviewed (Jurgens et al. 2012, Bankar et al. 2013a). Processes were developed in recent years, and some of the studies focused on lignocellulosic feedstocks utilization, hydrolysis, and fermentation methods are summarized in Table 3.

Various studies were conducted to improve the problems with biobutanol production. Efficient pretreatment and enzymatic hydrolysis method could improve the utilization of lignocellulosic biomass (Silverstein et al. 2007, Alvira et al. 2010). As it is shown in Table 3, pretreatment with an acidic thermochemical condition is often employed in the process of biobutanol production, but the removal of inhibitors is usually necessary. Potential inhibitors in fermentation media could result in incomplete sugar utilization by *Clostridium*, and affect the solvent production. Similar with ethanol fermentation by yeast, the toxic compounds produced during the pretreatment affects the microbial cell growth, glycolytic and fermentative enzymes in the central metabolic pathways (Taylor et al. 2012, Ibraheem and Ndimba 2013). These compounds also cause the DNA degradation and extensive membrane disruption that allow the release of proteins, RNAs, ATP, ADP, and ions outside from the

**Table 3.** Recent studies on biobutanol production from various feedstocks.

Feedstocks	Hydrolysis methods	Strain used	Inhibitors removal	ABE (g/L)	Yield (g/g)	Fermentations	References
Wheat bran	Dilute sulfuric acid	<i>C. beijerinckii</i> ATCC 55025	Overliming with Ca(OH) <sub>2</sub>	11.8	0.32	Batch	Liu et al. 2010
Wheat straw	Dilute sulfuric acid and enzyme	<i>C. beijerinckii</i> P260	No	25.0	0.42	Batch	Qureshi et al. 2007
Wheat straw	Dilute sulfuric acid and enzyme	<i>C. beijerinckii</i> P260	No	16.6	0.44	SSF and fed-batch with gas stripping	Qureshi et al. 2008a
Wheat straw	Dilute sulfuric acid and enzyme	<i>C. beijerinckii</i> P260	No	21.4	0.41	SSF with gas stripping	Qureshi et al. 2008b
Wheat straw	Alkaline peroxide and enzyme	<i>C. beijerinckii</i> P260	Electrodialys	22.2	0.42	Batch	Qureshi et al. 2008c
Barley straw	Dilute sulfuric acid and enzyme	<i>C. beijerinckii</i> P260	Overliming with Ca(OH) <sub>2</sub>	26.6	0.43	Batch	Qureshi et al. 2010a
Corn fiber	Dilute sulfuric acid	<i>C. beijerinckii</i> BA101	Ca(OH) <sub>2</sub> and XAD-4 resin	9.3	0.39	Batch	Qureshi et al. 2008d
Corn stalk	Steam-exploded and enzyme	<i>C. beijerinckii</i> ATCC 55025	Washing	5.7	0.16	Batch	Mu et al. 2011
Corn stover	Alkaline and enzyme	<i>C. acetobutylicum</i> ATCC 824	Washing	11.2	-	Batch	Zhang et al. 2014
DDGS	AFEX and enzyme	<i>C. beijerinckii</i> BA101	No	10.4	0.34	Batch	Ezeji and Blaschek 2008
Spent liquor of spruce	SO <sub>2</sub> -ethanol-water pulping	<i>C. acetobutylicum</i> DSM 792	Evaporation, steam stripping, liming, oxidation	8.8	0.20	Continuous	Survase et al. 2011
Sugar maple wood	Hot water extraction with sulfuric acid	<i>C. acetobutylicum</i> ATCC 824	Overliming with Ca(OH) <sub>2</sub>	11.0	-	Batch	Sun and Liu 2012
Wood pulping hydrolysate	Sulfuric acid	<i>C. beijerinckii</i> CC101	Resin and evaporation	17.7	0.32	Batch with gas stripping	Lu et al. 2013

cytoplasm resulting in reduction in cell replication rate, damaged polynucleotides, decreased intracellular pH, increased cell turgor pressure, decreased ATP production, and diminished proton motive force and nutrient transport (Ibraheem and Ndimba 2013, Abdehagh et al. 2014, Baral and Shah 2014). Formic acid is a critical fermentation inhibitor for *C. acetobutylicum* at the concentration above 0.4 g/L, but the solvent production by *C. beijerinckii* was not affected at the tested concentration range of formic acid (0–1.0 g/L) (Cho et al. 2012). Wang et al. (2011) found that addition of 1 mM formic acid could trigger “acid crash” in the fermentation of corn mash medium by *C. acetobutylicum*. The *C. beijerinckii* could secrete formate dehydrogenase which catalyzes the oxidation of formate to carbon dioxide (Calusinska et al. 2010). Furfural and HMF below 1.0 g/L are not inhibitory to *C. beijerinckii* BA101, but could stimulate the growth of microorganism and ABE production (Ezeji et al. 2007a). Levulinic acid at 1 g/L did not affect the ABE fermentation by *C. beijerinckii* CC101 (Lu et al. 2013). Phenolic compounds such as p-coumaric and ferulic acids are more toxic than furfural and HMF (Palmqvist and Hahn-Hägerdal 2000, Ezeji et al. 2007a). Studies also found that sodium sulfate and sodium chloride formed during the neutralization process are toxic to *Clostridium* (Ezeji et al. 2007a, Qureshi et al. 2008b). Additionally, the coexistence of these compounds increases the inhibitory effect on fermentative process (Mussatto and Roberto 2004).

Traditional distillation is a common process used in ABE industry for butanol recovery. However, the low concentration of butanol in fermentation broth makes the process energy intensive, and not cost effective. Ezeji et al. (2004) suggested that if the butanol concentration increased from 10 to 40 g/L, a tremendous amount of energy can be saved. However, butanol exceeding 13 g/L is toxic to the butanol producing strains (Baral and Shah 2014). To overcome problems with the high cost of butanol recovery and the butanol toxicity to *Clostridium*, efficient *in situ* product recovery technologies such as gas stripping, pervaporation, and adsorption are developed (Ezeji et al. 2003a, Nielsen et al. 2009, Li et al. 2011, Lu et al. 2012, Xue et al. 2012, Chen et al. 2014). With gas stripping for *in situ* product removal, ABE production from wood pulping hydrolysate increased to 17.73 g/L, compared to 11.35 g/L in control experiment (Lu et al. 2013). Continuous ABE fermentation with *in situ* solvent recovery by pervaporation produced a highly concentrated condensate containing 89.11–160.00 g/L ABE (Li et al. 2014). Xue et al. (2013a) achieved a condensate containing 420.3 g/L butanol (532.3 g/L ABE) with a two-stage *in situ* gas stripping process in a fibrous bed bioreactor. Although these technologies could reduce the product inhibition and improve the solvent yield and productivity, none of them could be applied in industrial scale. Additional costs of capital investment on facilities and energy consumption of running these facilities would be the main reason (Xue et al. 2013b). In addition, the loss of products and nutrients, and fouling problems need to be improved.

According to the production capacity, ABE fermentation can be operated with batch, fed-batch or continuous mode. Batch fermentation is the most often used mode in ABE industry due to its flexible operation and easy control over the process. However, substrate and product inhibition, and downtime for cleaning, sterilizing and filling are obviously restricting the solvent productivity. The productivities of ABE batch fermentation from lignocellulosic biomasses were as low as 0.017–0.42 g/L h (Jurgens et al. 2012, Jang et al. 2012). Fed-batch fermentation can alleviate the substrate inhibition by feeding concentrated medium, but it must be operated with *in situ* removal of butanol to reduce the butanol toxicity to cells. On the other hand, continuous fermentation is more suitable for butanol production at large scale because it eliminates downtime and improves the productivity. The continuous fermentation of semidefined P2 medium containing 6% maltodextrin or glucose by *C. beijerinckii* BA101

improved the productivity by 470% over the traditional batch process (Formanek et al. 1997). Furthermore, application of immobilized cell culture and cell recycle reactors is known to increase cell density and reactor productivity (Lienhardt et al. 2002, Tashiro et al. 2005, Qureshi et al. 2005). Immobilization allows long survival time of cells (due to lack of mechanical agitation) in solventogenesis phase without frequent cell regeneration. By application of a fibrous bed bioreactor with *C. acetobutylicum*, the butanol yield increased by 20% with continuous fermentation of corn substrate (Huang et al. 2004). Recycling of butanol free effluent increased the sugar utilization to 100.7% in addition to a high productivity in a continuous immobilized cell (biofilm) plug-flow reactor (Lienhardt et al. 2002). Continuous two stage ABE fermentation with integrated solvent removal and immobilized cell produced 25.32 g/L ABE from glucose medium with the solvent productivity and yield of 2.5 g/L h and of 0.35 g/g, respectively (Bankar et al. 2012). A two stage immobilized column reactor with integrated liquid-liquid extraction solvent recovery module allowed long run system operation with high solvent productivity of 10.85 g/L h, that could be integrated to the wood based biorefinery to make it industrially feasible (Bankar et al. 2013b).

The butanol concentration varies depending on different feedstocks, processing methods, fermentation technologies, but also the *Clostridium* spp. Good fermentation performance of *C. beijerinckii* P260 was found with the hydrolysates of wheat straw and barley straw as substrates (Table 3). The fermentation performance of *Clostridium* can be improved using chemical mutagenesis, evolutionary engineering and metabolic engineering strategies (Lee et al. 2009, Green 2011, Lütke-Eversloh and Bahl 2011, Jang et al. 2012). A hyperbutanogenic strain *C. beijerinckii* BA101 obtained by chemical mutagenesis can produce 33 g/L ABE, of which 18 g/L is butanol (Ezeji et al. 2004). A *Clostridium beijerinckii* mutant RT66 with considerable inhibitor-tolerance produced 12.9 g/L ABE, and 9.3 g/L was butanol from non-detoxified hemicellulosic hydrolysate of corn fiber (Guo et al. 2013). Through repetitive evolutionary domestications, a butanol tolerant strain *C. acetobutylicum* T64 which can tolerate 4% (v/v) butanol was isolated, and it produced 15.3 g/L butanol compared to 12.2 g/L produced by the wild type strain (Liu et al. 2013). Some progress has also been made by genetic modification of *Clostridia*, for example, disruption of the acetoacetate decarboxylase gene *adc* in *C. acetobutylicum* EA2018 to block acetone formation significantly increased the butanol ratio from 70% to 80% (Jiang et al. 2009). Bankar et al. (2014) used a modified *C. acetobutylicum* DSM792 to produce an alcohol biofuel mixture isopropanol-butanol-ethanol (IBE) instead of ABE. Nowadays, the development of omics provides an effective study tool for understanding the physiology mechanism of *Clostridium*, and rational designs targeting individual genes, enzymes or pathways can be adopted for improving butanol production. On the other hand, genetic modification of heterologous microbe species such as *E. coli* and *S. cerevisiae* with butanol pathway is also an alternative way to improve butanol yield without the production of the byproducts acetone and ethanol.

## 2.5 Study aims and objectives

This aim of this study was to develop processes for efficient biobutanol production using barley straw as a lignocellulosic substrate. Dilute acid pretreatment was employed to solubilize hemicellulosic sugars mostly xylose in barley straw from cellulosic biomass. The hemicellulosic hydrolysate and cellulosic stream was separately used in ABE fermentation. For efficient utilization of hemicellulosic hydrolysate in ABE fermentation, co-fermentation

of hemicellulosic hydrolysate with starch-based biomass, in which the production medium contains pentose derived from hemicellulose and starch or starch-based glucose, was investigated. The pretreated solid residues contains mostly the cellulosic biomass that was used for enhanced enzymatic hydrolysis by synergistic cooperation of cellulases with xylanase and surfactants to produce fermentable sugars efficiently, and followed by the ABE fermentation. The specific objectives of this study were as follows:

- i. To investigate dilute sulfuric acid pretreatment followed by enzymatic hydrolysis for simultaneously conversion of barley straw and grain to fermentable sugars (Article I);
- ii. To study the co-fermentation of hemicellulose and starch-based biomass for efficient pentoses utilization in ABE production, that could potentially reduce production costs, and relieve the fermentation inhibiting effect of sugar degradation products (Article II);
- iii. To improve the sugar production from dilute acid pretreated barley straw by additive xylanase and surfactants in enzymatic hydrolysis for ABE fermentation (Article III);
- iv. To investigate the feasibility of ABE fermentation of acid stored green barley silage (Article IV).

### **3 MATERIALS AND METHODS**

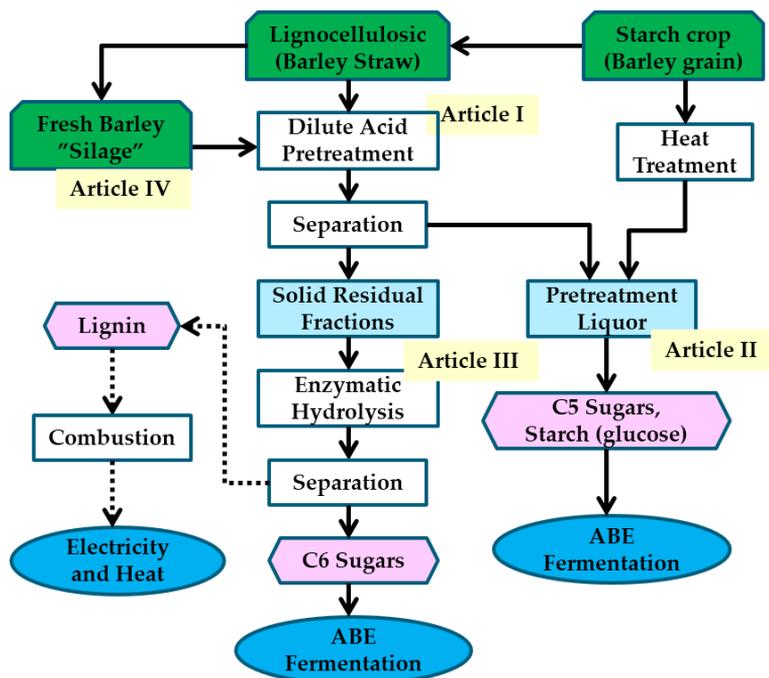
#### **3.1 Materials**

Barley straw and grain were harvested in 2011 from a field in North Karelia, Finland (Article I, II and III). The fresh forage barley was collected in 2012 from a field in North Karelia, Finland (Article IV). The fresh barley which was harvested when the leaves and grains were green is defined as green barley. The fresh barley which was harvested when the leaves and grains were yellow is defined as yellowish barley.

#### **3.2 Experimental design**

The central idea of this study was to efficiently use barley straw as a lignocellulosic substrate for biobutanol production (Fig. 2). The hemicellulosic and cellulosic streams were separated with dilute sulfuric acid pretreatment. The hemicellulosic hydrolysate was co-fermented with starch-based biomass for ABE production. There were two co-fermentation processes:

- I) Barley straw was mixed with barley grain, and the mixture was pretreated with dilute acid pretreatment. The sugars released mainly from hemicellulose and starch into the pretreated hydrolysate of the mixture were co-fermented for biobutanol production. In Article I, the effect of dilute acid pretreatment conditions on sugar recovery from the mixture of barley straw and grain was investigated.
- II) The pretreated barley straw hydrolysate was co-fermented with gelatinized barley grain slurry for biobutanol production. The two processes were compared for efficient hemicellulosic sugars utilization in Article II.



**Figure 2.** Simplified process flow diagram of experimental design (Lignin stream was not studied).

The cellulosic stream (SRFs) was used for efficient enzymatic hydrolysis by supplementing xylanase and surfactants in the hydrolysis by cellulase, and followed by ABE fermentation (Article III). In Article IV, fresh barley silage was used as an example of the co-fermentation process, and the feasibility of the pretreatment liquor of fresh barley silage for biobutanol production was investigated.

### 3.3 Pretreatment

In Article I, dilute sulfuric acid pretreatments were operated at 121°C, 1.1 bar in tubes with a working volume of 20 mL. A mixture of barley straw and grain, and grain and straw separately were pretreated with different concentrations of sulfuric acid (0.5, 1.0, 1.5 and 2.0%, w/v), and different lengths of reaction time (15, 30, 60 and 120 min with 1.0% sulfuric acid), respectively. The dry matter (DM) loadings for three substrates were 10%, 6% or 4% (w/v), respectively. After pretreatment, the pretreated hydrolysates (PHs) were separated from the solid residual fractions (SRFs). The pH of pretreated hydrolysates was measured with a pH metre. The sugars in PHs were measured with gas chromatography-mass spectrometry (GC-MS), and the sugars recovery was also calculated. The chemical composition in SRFs was analyzed.

In Article II, in process I, a mixture of barley straw and grain (10% DM loading, w/v) that contains 6% grain and 4% straw was heat treated with 1.0 and 1.5% sulfuric acid at 121°C, 1.1 bar for 60 min in triangle glass flask with a working volume of 200 mL. The

mixture hydrolysates containing mainly glucose degraded from starch of grain and xylose from hemicellulose of straw were used as medium for ABE fermentation. Additionally, two different proportions of grain and straw were tested for hydrolysis with 1.0% sulfuric acid and fermentation. The two proportions of grain and straw were: 1) 4% grain and 6% straw; and 2) 2% grain and 8% straw. The pretreated hydrolysates from the two mixtures were supplemented with xylose, as the concentration of additional xylose were 10 and 15 g/L respectively, to keep a suitable total sugar concentration for ABE fermentation.

In process II, the straw (7% DM loading, w/v) was heat treated with dilute acid (1.0% and 1.5% sulfuric acid, w/v) at 121°C, 1.1 bar for 60 min in triangle glass flask with a working volume of 200 mL. The grain slurry was gelatinized at 121°C for 60 min, and mixed with pretreated straw hydrolysates in a ratio of 1:1 (v/v). The grain content in the mixture was 6% (w/v). The mixture containing mainly gelatinized starch from grain and xylose from hemicellulose of straw was used as media for ABE fermentation. In further experiments, two gelatinized grain slurry samples with lower amount of grain were mixed with straw hemicellulosic hydrolysates (treated with 1.5% sulfuric acid). The final grain content was 4% and 2% (w/v), respectively. For keeping a suitable fermentation sugar level, 10 and 20 g/L xylose was supplemented into the two mixtures, respectively. The fermentations in process I and II were all compared with the fermentation of straw hemicellulosic hydrolysate (treated with 1.0% sulfuric acid) supplemented with 30 g/L xylose.

In Article III, straw was pretreated with different concentrations of sulfuric acid (0.5, 1.0, 1.5 and 2.0%, w/v) in 60 min in triangle glass flask with a working volume of 200 mL. The chemical composition in SRFs was analyzed.

In Article IV, the fresh barley sprayed with a mixture of H<sub>2</sub>SO<sub>4</sub>, HCl and H<sub>2</sub>O (1:1:1) was stored in closed container for six month. The procedure mimics the animal feed harvesting procedure commonly applied in the forage storage in mixed farming in Finland (Typpiomavaraistoimikunnanmietintö 1951). The green and yellowish silage was mixed with water or H<sub>2</sub>SO<sub>4</sub> with 6.25% (w/w) DM loading in triangle glass flask with a working volume of 200 mL, and then milled into slurry by using a stirrer (Bamix, Switzerland). The pretreatment was operated at 121°C, 1.1 bar for 60 min. The PHs defined as pretreatment liquor was used for analysis of sugars and sugar degradation products, and ABE fermentation. The pretreated SRFs defined as remaining lignocellulosic fractions were used for enzymatic hydrolysis.

### 3.4 Enzymatic hydrolysis

Enzymes Celluclast 1.5L, Novozyme 188, and endo-1,4-β-Xylanase (Mexico) were purchased from Sigma-Aldrich. PEG 4000 (Fluka, Germany) and Tween 80 purchased from Sigma-Aldrich were used as surfactants.

In Article I and IV, enzymatic hydrolysis of SRFs with 2% (w/v) DM loading was carried out in tubes with a working volume of 3 ml in 0.05 M sodium acetate buffer (pH 5.0). Hydrolysis was performed in a shaker with stirring at 200 rpm and 50°C for 48 h. In the hydrolysis, Celluclast 1.5 L (10 FPU/g biomass) and Novozyme 188 (200 nkat/g biomass) were added. In Article III, enzymatic hydrolysis of pretreated straw and microcrystalline cellulose with 2, 4, 6, 8% (w/v) DM loading was carried out in tubes with a working volume of 3 ml in 0.05 M sodium acetate buffer (pH 5.0). Enzymatic hydrolysis of pretreated straw with 8% (w/v) DM loading for fermentation was carried out with 100 ml working volume in

250 ml screw cap bottles. Prior to hydrolysis, 10 FPU/g biomass of Celluclast 1.5 L and 400 nkat/g biomass of Novozyme 188 were added as one dosage of CEL (Celluclast 1.5L and Novozyme 188) to the slurry for enzymatic hydrolysis. Different dosages of xylanase (0.5, 1, 2, and 5 g/100g DM) and surfactants (0.5, 1, 2, 5, and 10 g/100g DM) were added to the slurry to investigate synergistic cooperation with cellulase preparation.

After enzymatic hydrolysis, the samples were boiled for 10 min to stop the enzymatic reaction, and then centrifuged for 10 min at 12,000 rpm. The supernatant was collected for reducing sugar analysis.

### 3.5 Microorganism cultivation and fermentation

*Clostridium acetobutylicum* DSM 1731 was obtained from DSMZ, Braunschweig Germany (German Collection of Microorganisms and Cell Cultures). Freeze-stored culture was activated in 50 mL of Reinforced Clostridial Medium (Hirsch and Grinsted 1954) for 14–16 h. Then 1 mL of active culture was inoculated into 50 mL of sterilized pre-fermentation P2 media prepared in a 125 mL screw-capped bottle. The pre-fermentation P2 media contained glucose 30 g/L and yeast extract 1 g/L. Before inoculation, each of the filter-sterilized stock solutions (Buffer:  $\text{KH}_2\text{PO}_4$ , 50 g/L;  $\text{K}_2\text{HPO}_4$ , 50 g/L; ammonium acetate, 220 g/L; Mineral:  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 20 g/L;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 1 g/L;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 1 g/L; NaCl, 1 g/L; and Vitamin: para-aminobenzoic acid, 0.1 g/L; thiamin, 0.1 g/L; biotin, 0.001 g/L) was added into the P2 media. The culture was allowed to grow for approximately 16 h at 37°C before inoculation into the ABE production media.

ABE fermentations were conducted in 125 mL screw-capped bottles containing 50 mL media. In Article II, the media contained PHs of straw or a mixture of straw and grain, and yeast extract 1 g/L. The pure sugar media contained xylose or glucose-xylose mixture 50 g/L and yeast extract 1 g/L, and prior to the inoculation, each of the filter-sterilized stock solutions (buffer, mineral and vitamin) was added to the media. The pH of the media was adjusted to 6.5 with NaOH prior to fermentation. In Article III, the enzymatic hydrolysate was used as the media in which yeast extract 1 g/L, 0.5 mL each of the filter-sterilized stock solutions (buffer, mineral and vitamin) was added. The pH of the media was adjusted to 6.5 with  $\text{Ca}(\text{OH})_2$  prior to fermentation. In Article IV, the media contained 30 mL pretreatment liquor of green or yellowish barley, 20 mL gelatinized grain slurry and yeast extract 1 g/L. The pH of the media was adjusted to 6.5 with NaOH prior to fermentation. The media were purged with  $\text{N}_2$  for 10 min to maintain an anaerobic condition and sterilized at 121°C for 20 min. Fermentation started at 37°C when inoculated into the *C. acetobutylicum* DSM 1731 culture (10%, v/v). The fermentation samples were taken at 24 h intervals.

### 3.6 Chemical analysis

The chemical composition of raw materials and pretreated SRFs was measured following the standard protocol of the National Renewable Energy Laboratory (NREL) (Sluiter et al. 2006). In Article I and IV, 30 g materials were treated with 0.3 mL 72%  $\text{H}_2\text{SO}_4$  for 1 h at 30°C in 10 mL tubes, and then diluted to 4%  $\text{H}_2\text{SO}_4$  by adding 8.4 mL deionized water. In Article III, 300 g materials were treated with 3 mL 72%  $\text{H}_2\text{SO}_4$  for 1 h at 30°C in 100 mL triangular flask, and then diluted to 4%  $\text{H}_2\text{SO}_4$  with 84 mL deionized water. The diluted slurry was autoclaved at 121°C for 1 h, and then was neutralized with solid  $\text{CaCO}_3$  to pH 4–5. The slurry was

centrifuged for 10 min at 12,000 rpm, and the supernatant was collected for sugar analysis by GC-MS (Article I and IV) and Nuclear Magnetic Resonance (NMR) (Article III).

In Article I and IV, the sugars in PHs and compositional samples of SRFs were identified and quantified by GC-MS. The starch content in raw materials and fermentation residues was determined with Total Starch Assay Kit (Megazyme International Ireland Ltd.). Total reducing sugars in enzymatic hydrolysates were analyzed by the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959). The samples analyzed by GC-MS were centrifuged at 5,000 g for 10 min, and the supernatant was filtered through a 0.2  $\mu\text{m}$  sterilize syringe filter. The filtered samples were spiked with internal standard glucose- $^{13}\text{C}$  (0.2 mg/mL in methanol/water, 1/1), and evaporated to dryness. The samples were then treated with 80  $\mu\text{L}$  of methoxyamine hydrochloride solution (20 mg/mL) in pyridine for 90 min at 37  $^{\circ}\text{C}$ . Additionally, 80  $\mu\text{L}$  MSTFA was added and samples were incubated during silylation for another 60 min at the same temperature. The sugars were analyzed by GC-MS (Agilent 6890N with 5973 MS, Agilent Technologies, Palo Alto, CA, USA) with split injection (20:1) onto a Rxi-5Sil MS column (30 m  $\times$  0.25 mm  $\times$  0.25  $\mu\text{m}$ , Restek, USA). The temperatures of the injection port and the transfer line were 260 $^{\circ}\text{C}$  and 280 $^{\circ}\text{C}$ , respectively. The helium flow rate was 1 mL/min. Oven temperature was held at 70 $^{\circ}\text{C}$  for 1 min and increased at 5  $^{\circ}\text{C}/\text{min}$  until 320 $^{\circ}\text{C}$ , which was held for 3 min. The MS data were recorded in the mass range of 83–500 m/z. The analyses were identified by comparison with authentic standards.

In Article II, the sugars (glucose, xylose and arabinose), solvents (acetone, butanol, ethanol, acetic acid, butyric acid), and other compounds (furfural, HMF, formic acid and lactic acid) in the samples were analyzed with NMR. The NMR spectra for quantification of these compounds were recorded on a Bruker AVANCE 500 DRX NMR spectrometer equipped with a 5 mm QNP SB probe. Above mentioned compounds were identified from routine two-dimensional proton-proton and proton-carbon correlated spectra. In the case of lactic acid, verification was made by adding pure compound to a studied NMR sample. Quantitative  $^1\text{H}$  NMR spectra were collected with water presaturation (zgcprr) by using a 90 $^{\circ}$  pulse angle, 48 dB presaturation power, 40 s relaxation delay, and 16 scans at 300 K. Prior to the NMR measurements, 200  $\mu\text{L}$  of sample liquid was transferred to a 5 mm NMR tube followed by addition of deuterium oxide ( $\text{D}_2\text{O}$ , 275  $\mu\text{L}$ ) and 3-(trimethylsilyl)-propionic- $\text{d}_4$  acid (25  $\mu\text{L}$ , 20 mM) in  $\text{D}_2\text{O}$  as an internal standard of known concentration.

The NMR spectra for quantification of sugars (glucose, xylose and arabinose) in Article III was recorded on a Bruker AVANCE IIIHD 600 NMR spectrometer equipped with a 5 mm Prodigy TCI cryoprobe. Above mentioned compounds were identified from routine two-dimensional proton-proton and proton-carbon correlated spectra.  $^1\text{H}$  NMR spectra for quantification of the compounds were collected with water presaturation (noesygppr1d) by using a 90 $^{\circ}$  pulse angle, 25 Hz presaturation field, 5.8 s recycle time, 10 ms mixing time, and 4 scans at 295 K. Prior to the NMR measurements, 200  $\mu\text{L}$  of sample liquid was transferred to a 5 mm NMR tube followed by addition of deuterium oxide ( $\text{D}_2\text{O}$ , 300  $\mu\text{L}$ ) and 3-(trimethylsilyl)-propionic- $\text{d}_4$  acid (25  $\mu\text{L}$ , 20 mM) in  $\text{D}_2\text{O}$  as an internal standard of known concentration. In the sugars  $^1\text{H}$  NMR spectrum, the ring O-CH(OH) signal give rise to two doubles due to rapid equilibrium between equatorial and axial forms. In the case of glucose ca. 64% is in b-form (axial) and the rest in a-form, which values are similar to literature values. Aldehyde form (straight chain form) was not observed. Similar situation was with other sugars, xylose and arabinose. Concentration of each sugar is calculated based on these integral sums of O-CH (OH) protons which are compared to integral of standard with known amount.

Fermentation samples for ABE and acids (acetic acid and butyric acid) analysis in Article III and IV were analyzed using gas chromatography (Agilent 6890 N) equipped with a flame ionization detector. Restek Stabilwax-DA column (30 m x 0.32 mm x 1  $\mu$ m, Agilent Technologies, Finland) was used. The split ratio was 30:1, and the injector and detector temperatures were 250°C. The injector volume was 0.5  $\mu$ L. Oven temperature was held at 40°C for 2 min and increased at 5 °C/min until 245°C, which was held for 2 min. Isobutanol was used as an internal standard. The total reducing sugars in fermentation samples were analyzed with DNS method.

### 3.7 Calculations

In Article I, the equations for carbohydrate recoveries were expressed as below.

$$\begin{aligned} \text{Total carbohydrates recovery (\%)} \\ &= \frac{\text{Starch, glucan, xylan and arabinan in PHs and SRFs (g)}}{\text{Total raw biomass (g)}} \\ &\times 100 \end{aligned} \quad (1)$$

$$\begin{aligned} \text{Glucose recovery in PHs (\%)} \\ &= \frac{\text{Glucose in PHs (g)} \times 0.9}{\text{Theoretical glucan in raw biomass (g)}} \times 100 \end{aligned} \quad (2)$$

$$\begin{aligned} \text{Xylose recovery in PHs (\%)} \\ &= \frac{\text{Xylose in PHs (g)} \times 0.88}{\text{Theoretical xylan in raw biomass (g)}} \times 100 \end{aligned} \quad (3)$$

$$\begin{aligned} \text{Glucose recovery in SRFs (\%)} \\ &= \frac{\text{Glucose in enzymatic hydrolysate of SRFs (g)} \times 0.9}{\text{Theoretical glucan in raw biomass (g)}} \times 100 \end{aligned} \quad (4)$$

The pretreatment conditions were expressed as the combined severity (CS), which is a function of reaction time, temperature and pH. It was defined (Chum et al. 1990) as:

$$CS = \log R_0 - pH \quad (5)$$

$$R_0 = t \times \exp [(T_H - 100^\circ\text{C})/14.75]$$

Where t is the reaction time in minutes and  $T_H$  is the hydrolysis temperature in °C.

In Article III, the equations for sugar yields from enzymatic hydrolysis were:

$$\text{Glucose yield (\%)} = \frac{\text{Glucose in enzymatic hydrolysate (g)} \times 0.9}{\text{Glucan in pretreated substrates (g)}} \times 100 \quad (6)$$

$$\text{Xylose yield (\%)} = \frac{\text{Xylose in enzymatic hydrolysate (g)} \times 0.88}{\text{Xylan in pretreated substrates (g)}} \times 100 \quad (7)$$

In Article IV, the equation for total sugar yields from green and yellowish barley were:

$$\begin{aligned} & \text{Toal sugar recovery (\%)} \\ &= \frac{\text{Total reducing sugars in enzymatic hydrolysate (g)} \times 0.9 + \text{pretreatment liquor (g)} \times 0.88}{\text{Glucan, xylan and arabinan in pretreated substrates (g)}} \\ & \times 100 \end{aligned} \quad (8)$$

## 4 RESULTS AND DISCUSSION

### 4.1 Biobutanol production from hemicellulosic biomass in barley straw (Article I, II)

#### 4.1.1 Xylose recovery from barley straw and a mixture of barley straw and grain

With the increasing acid concentration from 0.5 to 1.5%, and the increasing reaction time from 15 to 120 min, the concentrations of xylose released into the pretreated straw hydrolysates were increased from 4.6 to 13.0 g/L, and the xylose content in the mixture hydrolysates were increased from 5.8 to 12.3 g/L (Article I, Table 2). The pretreatment with 2.0% sulfuric acid resulted in a lower xylose concentration. When the pretreatment conditions were expressed as the combined severity (CS, Article I, Fig. 3), the results suggest that when the pretreatment condition is more severe, more xylose was released from lignocellulosic biomass, however, with higher severity pretreatment, sugar degradation products were possibly formed. This is in agreement with previous studies of pretreatment of barley straw and wheat straw (Kabel et al. 2007, Panagiotopoulos et al. 2011). However, the longer time (120 min) did not result in low concentration of xylose, which implies that the sulfuric acid concentration more severely affects xylose recovery than reaction time. Thus, the CS factor could suggest the gross trend of xylose recovery, but could not predict xylose recovery at elevated acidic conditions.

Xylan is the main hemicellulosic constituent in barley straw and the mixture of barley straw and grain (Article I, Table 1). After the pretreatments, xylose is the primary hemicellulosic sugar in the pretreated hydrolysates (Article I, Table 2). The optimal xylose 12.43 g/L and 12.31 g/L was released from barley straw and the mixture pretreated both with 1.5% sulfuric acid for 60 min. The amount of xylose released from straw was approximately 100% of the theoretical xylose, but the xylose released from the mixture only accounts for 57% of the theoretical xylose. The xylose recovery from the mixture was lower than that from straw, but similar concentrations of xylose were obtained. This is probably due to the inaccessibility of acids to non-starch polysaccharides like barley husks that caused by mixing with starch. It was reported that in pretreatment of barley husks, the yield of extracted arabinoxylan was low due to high amount of starch dispersed in the aqueous phase (Persson et al. 2009). However, no significant amounts of xylan were obtained in the SRFs (Article I, Table 2). This indicates that xylan was probably decomposed into xylo-oligomers in pretreated hydrolysate.

As described in Article II, mixed feedstocks from a variety of sources would be utilized in a biorefinery. The conversion of both starch and lignocellulose in these materials simultaneously to fermentable sugars could leave out the biomass separation step, and

potentially reduce the production costs. In this study, although lower xylose recovery was obtained, the overall glucose recovery from the pretreated hydrolysate and SRFs of the mixture was approached to 96%. According to previous studies, dilute sulfuric acid pretreatment is highly efficient in hydrolysis of hemicellulose and starch to its monomeric units, rendering the cellulose more available in the mixture of starch and lignocellulosic materials (Agbor et al. 2011, Hoseinpour et al. 2013). The mixture hydrolysate with a relatively higher sugar concentration than that in straw hydrolysate would be a good substrate for the following ABE fermentation. The concentration of inhibitors in mixture hydrolysate was lower than that in straw hydrolysate (Article II, Fig. 2). This can most probably be explained with the mixing of straw and grain; the increase in dry matter loading might decrease the relative  $H_3O^+$  ion content, which is directly proportional to biomass hydrolysis and degradation. For the subsequent fermentation of hemicellulosic hydrolysate, that of lower amount of inhibitors would be more favorable.

#### 4.1.2 *ABE fermentation of pretreated hydrolysate of straw*

Fermentation of straw hemicellulosic hydrolysate (pretreated with 1.0% sulfuric acid) supplemented with 30 g/L xylose only produced 1.1 g/L ABE, in which there were 0.68 g/L butanol (Article II, Fig 4). After fermentation, 29.4% pentoses were utilized. However, by addition of 6% heat treated grain into the hemicellulosic medium, the co-fermentation of straw hydrolysate and grain slurry produced 13.5 g/L ABE as the highest, which contained 7.8 g/L butanol. In this fermentation, 97% pentoses were utilized (process II, Article II, Table 1). The ABE concentration was also higher than control fermentation of 6% grain slurry. The proportion of grain and straw hydrolysate was changed by reducing grain content to 4% and 2%, and supplementing 10 and 20 g/L xylose. The fermentations resulted in slightly lower ABE concentration, and the butanol concentrations were similar. The pentoses utilization was 84.4 and 75.8%, respectively.

The poor fermentability of straw hemicellulosic hydrolysate is probably due to the inhibitory effects of sugar degradation products (Article II, Fig. 2). Qureshi et al. (2010a) found that barley straw hydrolysate pretreated with dilute sulfuric acid may have been toxic to the fermentation culture. The co-fermentation of hemicellulosic hydrolysate and grain slurry diluted the concentration of inhibitory chemicals in the medium, and improved the ABE production significantly. It was reported that dilution of the barley straw hydrolysate pretreated with dilute sulfuric acid significantly improved the butanol production yield (Qureshi et al. 2010a). Furthermore, the mixture of grain slurry and straw hydrolysates which were pretreated with 1.0% and 1.5% sulfuric acid showed similar fermentability. This suggests that the pretreatment conditions could be more severe for releasing more pentoses for ABE fermentation (Article II, Fig. 5).

For the onset and maintenance of ABE production, excess sugars are needed in the fermentation media (Ezeji and Blaschek 2008). In this study, straw hemicellulosic hydrolysate contains a low concentration of sugars, and for efficient ABE fermentation, it must be concentrated, and this may introduce an additional cost. The co-fermentation of hemicellulosic hydrolysate and grain slurry could increase the sugar concentration for fermentation by adjusting the starch content, which could keep the ABE production more efficient. It has been found in previous studies that it was beneficial for both first generation and second generation ethanol and biohydrogen production to mix wheat grain hydrolysate with wheat straw hydrolysate (Erdei et al. 2010, Panagiotopoulos et al. 2013).

#### 4.1.3 ABE fermentation of pretreated hydrolysate of a mixture of barley straw and grain

Fermentation of mixture hydrolysate pretreated with 1.0% sulfuric acid (M1.0) produced 11.3 g/L ABE, of which 7.4 g/L was butanol in 96 h (process I). During the fermentation, almost all glucose was consumed, but only 19.0% of pentoses were consumed (Article II, Fig. 1). Fermentation of mixture hydrolysate pretreated with 1.5% sulfuric acid (M1.5) showed a lag phase of approximately 24 h before accumulation of a significant amount of ABE, and produced lower concentration of butanol and ABE. During the fermentation, the glucose was consumed slower than that in the fermentation of M1.0, and 2.8 g/L (24.9%) of pentoses were utilized (Article II, Fig. 1 and Table 1). Although more sugars were released in M1.5 during the pretreatment (Article II, Table 1), poor fermentability of M1.5 was observed (Article II, Fig. 1). This suggests that pretreatment with 1.5% sulfuric acid cause harmful effect on ABE fermentation, therefore the pretreatment with lower concentration of sulfuric acid would be more favourable in this process.

The fermentations showed the feasibility to produce butanol by co-fermentation of mixed sugars derived from hemicellulose and starch (pretreated with 1.0% sulfuric acid). It could increase the sugar concentration in fermentation media and achieve a higher ABE concentration when compared with the fermentation of straw hydrolysate alone. However, only few pentoses were utilized. A possible reason is that a high amount of glucose may inhibit xylose consumption in ABE fermentation by *C. acetobutylicum* ATCC824 (Ounine et al. 1985, Fond et al. 1986). When the glucose concentration in the mixture hydrolysate was reduced by decreasing the grain content in the pretreatment, the hydrolysate derived from biomass contains a moderate amount of grain (4%) resulted in similar ABE concentration with the hydrolysate contained 6% grain, and relatively higher xylose utilization (Article II, Table 3). This is in agreement with the results obtained by using sugar mixture medium (Article II, Table 3). Thus, for improved xylose utilization, biomass that contains a moderate amount of starch would be favorable for utilization in this process.

Although the fermentation of pretreated hydrolysate from biomass containing starch and lignocellulose provided a feasible process for utilization of hemicellulosic sugars, a mass of acid and alkali used for hydrolysis and neutralization would be a significant cost factor in large scale biofuel production systems. In contrast, co-fermentation of straw hydrolysate and grain slurry (process II) proceeds readily on hemicellulosic hydrolysates and grain slurry which needs not necessarily to be saccharified with enzymes. It simplifies the process design, and could facilitate the more efficient utilization of hemicellulosic hydrolysates. In the future, lignocellulosic feedstocks would be the main supply for large scale biofuels production. The co-fermentation of starch-based biomass with variety of lignocellulosic biomasses would be more favorable in a biorefinery.

## 4.2 Biobutanol production from cellulosic biomass in barley straw (Article III)

### 4.2.1 Enzymatic hydrolysis of pretreated straw

With an increasing acid concentration from 0.5% to 2.0%, the enzymatic hydrolysis of pretreated straw by using CEL (Celluclast 1.5 L and Novozyme 188) becomes more efficient (Article III, Fig. 1). However, considering that 2.0% sulfuric acid resulted sugar loss in the hemicellulosic hydrolysate, pretreatment with 1.5% sulfuric acid would be the preferable

condition for sugar release from both hemicellulose and cellulose (Article II). Hydrolysis of straw pretreated with 1.5% sulfuric acid by CEL resulted in a 53.2% glucose yield and a 36.2% xylose yield (Article III, Fig. 2). For efficient utilization of barley straw, this glucose yield is low, and even using a double enzyme dosage, the yield did not increase much (Article III, Fig. 1). As can be seen in Table 1 (Article III), dilute sulfuric acid pretreatment led to a reduction of hemicellulose and partial removal of glucan and lignin in straw. The residual hemicellulose and lignin are possibly affecting effectiveness of cellulose hydrolysis (Öhgren et al. 2007, Ge et al. 2014).

Xylan forms a complex network with cellulose by coating and connecting cellulose crystallites, and limiting the accessibility of cellulases towards cellulose (Ding and Himmel 2006, Dammström et al. 2009). The study of Penttilä et al. (2013) revealed that certain fraction of xylan remains tightly attached to cellulose fibrils, and certain fraction of xylan loosely forms a threedimensional structure throughout the lignocellulosic matrix. The presence of loosely bound xylan causes the increase of cellulose crystallinity and limits the hydrolysis of crystalline cellulose (Penttilä et al. 2013). Hydrolysis efficiencies of cellobiohydrolase I (CBHI), cellobiohydrolase II (CBHII) and endoglucanase II (EGII) were clearly inhibited by birchwood xylan in the hydrolysis of wheat straw, Avicel and nanocellulose (Zhang et al. 2012). The limitation can be overcome by addition of xylanase to remove the majority of loosely bound xylans (Bura et al. 2009, Várnai et al. 2011, Zhang et al. 2011, Penttilä et al. 2013). The synergistic interaction of the xylanase and cellulase enzymes could remove the blocking effect of xylan, increasing fiber swelling and fiber porosity, thereby improving cellulose accessibility (Hu et al. 2011a). However, in our study, the hydrolysis yields were somewhat improved by addition of xylanase in the enzymatic hydrolysis (Article III, Figs. 2 and S2), which indicates that the nonproductive binding of xylanase by the substrate and lignin, and enzyme denaturation or deactivation may have also occurred. Lignin has been considered to reversibly or irreversibly adsorb enzymes, which impairs their activity (Pareek et al., 2013).

By addition of PEG 4000 and Tween 80, a considerable increase of the hydrolysis yields was obtained (Article III, Figs. 2 and S3). Surfactants, especially non-ionic surfactants, could adsorb to lignin surface and significantly reduce unproductive binding of enzyme, thus enhancing the cellulose hydrolysis (Eriksson et al. 2002, Börjesson et al. 2007). The surfactants could also positively affect cellulase activity and enzymes stabilities (Ouyang et al. 2010). According to a study of Hsieh et al. (2013), the hydrolysis boosting effect of PEG is specific for exo-cellulase cellobiohydrolase (CBH I), but not for endoglucanase (EG). It has been reported that the degree of increased free cellulase activity obtained by PEG addition is in connection with the amount of phenolic hydroxyl groups in various substrates; the phenolic hydroxyl groups exposed on the lignin surface interact with PEG through hydrogen bonding, forming a layer of PEG on lignin surface, which prevents unproductive binding of cellulases on lignin (Sipos et al. 2011). Except for the reduction of unproductive binding of cellulase on lignin and cellulose, Li et al. (2012) confirmed that PEG 4000 prevents cellulase deactivation induced by cellulose, and promotes the removal of amorphous cellulose. Another mechanism related to PEG-water interaction rather than PEG-substrate or PEG-protein interactions was also proposed; the activity of enzyme on the substrate surface was increased by addition of PEG that is due to the increase of relaxation time of the liquid-phase water (Hsieh et al. 2013). However, surfactants do not consistently improve the enzymatic hydrolysis of pure cellulose, and it depends on the hydrolysis conditions (such as shaking speed, pH, and substrate concentration), cellulose structural features (DP of cellulose and surface morphology) and cellulase formulation (Zhou et al. 2015).

The combined application of xylanase and surfactants in the enzymatic hydrolysis resulted in further increase of the hydrolysis, and the glucose and xylose yields were both higher than that obtained with only one of them (Article III, Figs. 2 and S1). The maximum glucose yield of 86.9% and xylose yield of 70.2% was obtained by using CEL + xylanase + PEG 4000 (CXP, Article III, Fig 2). This can most probably be explained by the increased hydrolysis of residual xylan in pretreated straw. Surfactants could prevent the unproductive binding of xylanase to lignin, and also reduce the adsorption of xylanase on substrates (Ge et al. 2014). In the enzymatic hydrolysis of steam pretreated barley straw, xylanase supplementation maximized enzymatic hydrolysis yield, although cellulase preparations include some xylanase activity (García-Aparicio et al. 2007). It is notable that the use of xylanases with different hydrolytic patterns affects the efficiency of hydrolysis of lignocellulosic biomass. The xylanases with a carbohydrate-binding module could improve the enzymatic conversion of pretreated lignocellulosic materials by concentrating the enzyme on xylan rather than cellulose (Zhang et al. 2013). In short, the enhanced xylose production could contribute to the enzymatic hydrolysis of cellulose in lignocellulosic materials.

The benefits of the application of xylanase and PEG 4000 to the enzymatic conversion of pretreated barley straw containing residual xylan and lignin were clearly shown. The use of surfactants could reduce the nonspecific binding of enzymes to substrates and increase the enzyme stability that makes the effective use of enzymes, and makes the enzyme recovery possible. The addition of xylanase could remove xylan and increase the accessibility of cellulose, and this provides a possibility to employ a mild acid condition in pretreatment, which is usually recommended to balance sugar production and degradation. However, due to lack of industrial scale prices of surfactants and enzyme, it has not been possible to perform economic calculations on the feasibility of surfactant and xylanase addition.

#### 4.2.2 ABE fermentation of enzymatic hydrolysate

The enhanced enzymatic hydrolysis (With CXP) of barley straw (8% DM loading) pretreated with 1.5% sulfuric acid resulted in a hydrolysate containing 42.5 g/L total reducing sugars. The sugar concentration was apparently higher than that obtained only with CEL (25.7 g/L) (Article III, Fig. 3). The fermentation of enzymatic hydrolysate obtained with CXP produced 10.8 g/L ABE, of which 7.9 g/L was butanol (Article III, Fig. 4A). After fermentation, almost 95% of the total reducing sugars in the hydrolysate were consumed. The ABE and butanol yields calculated according to consumed sugars were 0.28 and 0.20 g/g reducing sugars, respectively. The concentrations of butanol and ABE were comparable with that (8.0 g/L butanol and 11.3 g/L ABE) obtained from the fermentation of pure glucose medium (Article III, Fig. 4B).

The ABE production from the enhanced enzymatic hydrolysate was compared with the fermentation of hydrolysate obtained with CEL, and increases in concentrations of ethanol, acetone, and butanol were obtained. This is due to the increase of sugar concentration in the fermentation medium. When ABE production yield was calculated according to the weight of raw materials, it increased from 93.8 to 135.0 g/kg pretreated straw by the combined application of xylanase and PEG 4000 in enzymatic hydrolysis. The efficiency of straw utilization was apparently increased. In previous studies, addition of surfactants showed positive effect on bioethanol production from lignocellulosic materials (Alkasrawi et al. 2003, Tu et al. 2009). Addition of Tween 20 increased the ethanol yield and reduced the time required to attain maximum ethanol concentration in SSF of steam-pretreated softwood, and

the amount of enzyme loading was reduced by 50% (Alkasrawi et al. 2003). Tween 80 improved the ethanol yield in separate hydrolysis and fermentation and SSF of steam exploded lodgepole pine, which was probably due to the reduction of nonproductive binding between enzyme and lignin (Tu et al. 2009). Additionally, Tween 80 showed slight improvement of both furfural and HMF consumption in SSF of steam exploded lodgepole pine, which could alleviate inhibitory effects on yeast fermentation (Tu et al. 2009). However, the positive effect of Tween 80 on ethanol production was not found in SSF of ethanol pretreated lodgepole pine (Tu et al. 2009). Although there are few studies related to the effect of surfactants on ABE fermentation, the benefit would be similar to ethanol fermentation, which improves sugar conversion and allows the enzyme recycling during the ABE production process. In a study of extractive ABE fermentation with non-ionic surfactants, Dhamole et al. (2012) found that a pluronic surfactant L62 (volume fraction of 6%) enhanced butanol yield by 225%, and the increase in butanol production might be attributed to the attachment of butanol to the monomers of L62, which probably relieved the butanol toxicity during fermentation.

In this study, the pretreated straw was separated from hemicellulosic hydrolysate and used for ABE fermentation that provides the possibility to improve sugar production by optimization of the enzyme system without effects from inhibitors. The hemicellulosic stream could be co-fermented with starch based biomass (Article II, process II). As studied earlier, separate hexose and pentose fermentation from steam-exploded corn stalk could improve the efficiency for butanol production (Mu et al. 2011).

### **4.3 Biobutanol production from acid stored green barley silage (Article IV)**

#### *4.3.1 Sugar recovery from green and yellowish barley silage*

By hydrothermal pretreatment and the following enzymatic hydrolysis of SRFs, 88% and 100% of the theoretical sugars were recovered from the green and yellowish barley silage, respectively (Article IV, Fig. 2). The sugar recovery from green barley silage was lower than that from yellowish barley silage, and this was probably due to the sugar degradation during the pretreatment. It was found that the concentrations of formic acid, levulinic acid and lactic acid formed as sugar degradation products in the pretreatment liquor of green barley silage were significantly higher than that in the pretreatment liquor of yellowish barley silage (Article IV, Table 2). However, these compounds were also formed in the pretreatment of yellowish barley silage with a 100% total sugar yield. This suggests that the sugar recoveries were overestimated possibly due to the underestimation of the carbohydrate analysis in the raw materials (Linde et al. 2007). When comparing the concentrations of sugars and sugar degradation products in the pretreatments with water, 1.0% and 1.5% sulfuric acid, there were no significant differences (Article IV, Table 2). This indicates that the acids used for preserving barley biomass have been strong enough for efficient pretreatment and enzymatic hydrolysis, by affecting the structure of biomass. The additional acid neither lead to the increase of sugar recovery nor the further degradation of sugars.

#### *4.3.2 ABE fermentation of pretreatment liquor*

The fermentation of pretreatment liquor of green barley silage supplemented with gelatinized grain produced 9.0 g/L ABE in 144 h, of which 6.2 g/L was butanol (Article IV, Fig. 3A).

The ABE and butanol yields were 0.28 and 0.20 (g/g monosaccharide), respectively. The fermentation of the pretreatment liquor of yellowish silage supplemented with gelatinized barley grain produced 10.9 g/L ABE solvents in 144 h, of which 7.3 g/L was butanol (Article IV, Fig. 3B). The ABE and butanol yields were 0.26 and 0.17 (g/g monosaccharide), respectively. The consumption of total reducing sugars in the fermentation of yellowish silage was faster than that in the fermentation of green silage, and ABE concentrations were higher (Article IV, Fig. 3).

The results suggest that the pretreatment liquor of green field biomass containing hemicellulosic sugars is a good substrate to co-ferment with starch-based biomass for ABE production. The remaining lignocellulosic fractions could be efficiently hydrolyzed as a promising feedstock for separately biobutanol production. On the other hand, the green and yellowish barley silage contains starch, which would be a possible replacement of media components that provide the nutrients for butanol fermentation. Ezeji et al. (2007b) found that it was not necessary to supplement a normal P2 medium for the fermentation of saccharified degermed corn due to presence of nutrients in the saccharified degermed corn. Better fermentability for yellowish silage was observed, this is probably not only due to the lower concentrations of sugar degradation products, but the higher starch content in yellowish barley silage may also provide more nutrients for ABE fermentation.

Green biorefinery is a concept to utilize fresh green biomass for the production of bio-based products including proteins, lactic acids, fibres and energy (Kromus et al. 2004). The results of this study showed the feasibility to produce biobutanol from green field biomass. In addition, the “silage” technique was successfully applied for biomass storage, which is one of the factors affecting the economic viability of lignocellulose utilization (Banerjee et al. 2010). The “silage” technique could improve the harvest efficiency and lower the harvesting cost. Furthermore, preserving biomass as silage avoids the field drying process, and the dry matter loss can be reduced to less than 5%, and also the substrate is more susceptible to enzymatic hydrolysis than dry-stored lignocellulosic biomass (Shinners et al. 2007).

#### 4.4 Comparison of biobutanol production to other studies

In recent years, various lignocellulosic feedstocks have been investigated for butanol production via ABE fermentation (Table 3). The concentrations of ABE and the production yields differ with different feedstocks, pretreatments, hydrolysis methods, fermentation technologies and *Clostridium* species. In our study, ABE and butanol yields ranged from 0.26 to 0.30 and 0.17 to 0.23 g/g monosaccharide, respectively (Article II, III and IV). The concentrations of butanol (6.2-7.8 g/L) and total ABE (9.0-13.5 g/L) were comparable to some of the studies with respect to butanol production using lignocellulosic materials, for example, sugar map wood, rice straw, sago pith residues, wheat straw and corn fiber (Sun and Liu 2012, Gottumukkala et al. 2013, Linggang et al., 2013, Bellido et al. 2014, Zhang et al. 2014). A typical butanol yield in ABE fermentation is 0.20 to 0.25 g/g (Lu et al. 2013). However, these yields and the concentrations of ABE are lower than those in some studies of butanol production by *C. beijerinckii* P260 from agricultural residues, like wheat straw (Qureshi et al. 2007, Qureshi et al. 2008abc), barley straw (Qureshi et al. 2010a), and corn stover (Qureshi et al. 2010b). The range of ABE concentration in these studies was from 16.6 to 26.6 g/L, and the yields were all greater than 0.4 g/g sugars. *C. beijerinckii* P260 is a

commercial strain which was used in South Africa in the early 1980s (Keis et al. 2001). Clostridium strain is the most important factor affecting the efficiency of biobutanol production process. A strain with improved phenotypes, such as non-spore forming, phase resistant, degeneration-deficiency, good feedstocks utilization ability, high butanol tolerance, high butanol yields and high ABE production is essential for commercial production of butanol from lignocellulosic feedstocks (Jones and Woods 1986). Strain improvement could be achieved by strain isolation, mutation and screening, and metabolic engineering technologies.

Lignocellulosic feedstocks such as agricultural and forest residues, energy crops, and industrial wastes have been extensively studied for butanol production (Table 3; Article II, Table 2). In order to accomplish large scale utilization of biomass feedstocks to produce biobutanol, mixed feedstocks from a variety of sources are probably utilized for ABE production. These are likely to include starch based materials such as corn, barley, sorghum, potato and sweet potato. Forage barley is an attractive biomass for converting both its starch and lignocellulose to fermentable sugars. It has also been regarded that barley would be a good supplement to corn biofuel production in anticipation of the commercialization of lignocellulosic biofuel (Nghiem et al. 2010). In Article I, various pretreatment conditions were studied and the optimum conditions were found to simultaneously convert lignocellulose and starch-based biomass to fermentable sugars. Combined with Article II (process I), a promising process for biobutanol production from the whole barley plants was developed. Shao et al. (2010) confirmed the feasibility of co-utilization of whole corn plants (grains plus cellulosic residues) using an ammonia-based (AFEX) pretreatment to increase bioethanol yield. It is widely believed that there would be significant cost savings from harvesting and processing whole plant rather than separately processing grain and the residues for production of biofuels (Kamm and Kamm 2007).

Clostridium is able to ferment both hexose and pentose sugars, however, xylose is not efficiently utilized, as observed in the fermentation of a mixture of glucose and xylose by *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052 (Gu et al. 2009, Hu et al. 2011b, Xiao et al. 2012). Gu et al. (2009) found that *C. acetobutylicum* ATCC824 utilized 86% of glucose within 40 h, whereas only 6% of xylose was consumed even after an elongated incubation time. In Article II (process II), the hemicellulosic hydrolysate was mixed with grain slurry, which promoted the efficiency of the xylose utilization during ABE fermentation. Although the promotion mechanism is not clear, the presence of starch could replace the nutrients supplementation, which would be cost-effective. Of course, other starch-based biomass, for example, food waste could also be investigated to further reduce the production costs. The cellulosic residues in this study were separately utilized for enzymatic hydrolysis, and the synergistic cooperation of cellulases with xylanase and surfactants significantly increased the sugar conversion and resulted in efficient feedstock utilization in ABE fermentation (Article III). The application of surfactants has been found to improve the bioethanol yield and productivity (Alkasrawi et al. 2003, Tu et al. 2009). Thus, the more study of effect of surfactants on ABE fermentation by Clostridium is needed. Overall, this study developed processes for the production of butanol as an alternative biofuel from agricultural feedstocks. In the future study, the processes could be investigated in wider range of materials, and the combined application with robust Clostridium strain, modern fermentation and butanol recovery technologies would be of interest.

## 5 CONCLUSIONS

The study elucidates the feasibility to efficiently utilize the hemicellulose and cellulose separately in barley straw for ABE fermentation. Fermentation of barley straw hemicellulosic hydrolysate alone resulted in a poor ABE production. By two processes of co-fermentation of hemicellulosic hydrolysate with starch-based biomass, much higher concentration of butanol and ABE was obtained than that in fermentation of hemicellulosic hydrolysate alone. In process I, co-fermentation of sugars derived from hemicellulose and starch in the mixture of barley straw and grain was studied. Reasonable sugar yields were obtained from a mixture of barley straw and grain with dilute sulfuric acid pretreatment and subsequent enzymatic hydrolysis. Although the mixture hydrolysate showed better fermentability for ABE production, only few pentoses were consumed. On the other hand, in process II, co-fermentation of straw hemicellulosic hydrolysate and gelatinized grain slurry facilitated pentoses utilization and produced more ABE. By comparison, process II could be applied to biofuel production from variety of lignocellulosic biomasses, and it is a favorable process for efficient utilization of hemicellulosic biomass.

For the utilization of cellulose in barley straw, the combined application of xylanase and surfactants in enzymatic hydrolysis of dilute sulfuric acid pretreated straw by cellulases was studied. The xylanase played the role of synergistic cooperation with cellulase in the hydrolysis with supplemented surfactants. A glucose yield of 86.9% and xylose yield of 70.2% was obtained by addition of xylanase and PEG 4000 in the enzymatic hydrolysis, which were considerably higher than results obtained with xylanase or PEG 4000 alone. The enzymatic hydrolysate showed good fermentability in ABE production, and was efficiently utilized as the ABE yield increased from 93.8 to 135.0 g/kg pretreated straw by combined application of xylanase and surfactants. In the context of biobutanol production, enhanced sugar production provides the possibility to get optimal sugars from biomass pretreated with mild conditions, and it could be applied to enzymatic hydrolysis with a high solid loading.

The use of green field barley silage was a typical application of co-fermentation of hemicellulosic biomass with starch-based biomass. By hydrothermal pretreatment and enzymatic hydrolysis, 88% and 100% of sugar recoveries were obtained from green and yellowish barley silage, respectively. The pretreatment liquor supplemented with gelatinized starch showed good fermentability for ABE production. In addition, the “silage” technique could be successfully applied for the storage of biomass containing lignocellulose and starch. The starch instead of media components may provide the nutrients for butanol fermentation.

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