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PREFACE

This Ph.D. thesis is the result of a research project conducted at the Section for Sustainable Biotechnology, Aalborg University Copenhagen, Denmark from August 2009 to August 2012. Associate Professor Hinrich Uellendahl was the main supervisor and Professor Birgitte K. Ahring was the co-supervisor. The Ph.D. research was financially supported by the Energy Technology Development and Demonstration Program of the Danish Energy Council, grant no.: 64009-0010.

The thesis is organized as a short summary in the beginning followed by a collection of journal manuscript, consisting of a review paper and four original research papers, ending with brief concluding remarks and future research. The individual manuscripts are presented with the journal for which the individual manuscripts are submitted to or intended as presented below.

I Biofuels and biomaterials production in a biorefinery – key features for sustainable and economically viable concepts.
Njoku, S. I. Uellendahl, H. Ahring, B. K.
Intended for submission to Sustainable Bioenergy Systems

II Pretreatment as the crucial step for a cellulosic ethanol biorefinery: Testing the efficiency of wet explosion on different types of biomass.
Njoku, S. I. Ahring, B. K. Uellendahl, H.
Published in Bioresource Technology.

III Tailoring wet explosion process parameters for the pretreatment of Cocksfoot grass for high sugar production.
Njoku, S. I. Ahring, B. K. Uellendahl, H.
In press in Applied Biochemistry and Biotechnology

IV Comparing oxidative and dilute acid wet explosion pretreatment of Cocksfoot grass at high dry matter concentration for cellulosic ethanol production.
Njoku, S. I. Uellendahl, H. Ahring, B. K.
Submitted to Energy Science and Engineering

V Production of ethanol from the hemicellulose fraction of cocksfoot grass using *Pichia stipitis*.
Njoku, S. I. Iversen, J. A. Uellendahl, H. Ahring, B. K.
Submitted to Sustainable Chemical Processes

Copenhagen, October 2012

Stephen Ikechukwu Njoku

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A journey is said to be easier and smooth when is made in a group and accordingly, this Ph.D. thesis is like that journey which took three years to complete with exciting company of several people who contributed to the completion of this Ph.D. thesis work for one way or another. Therefore, I wish to express my gratitude to the following.

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I am grateful to all the people at the Section for Sustainable Biotechnology, Aalborg University Copenhagen, Denmark, for contributing to a pleasant atmosphere especially the Ph.D. students and the laboratory technicians. I appreciate the priceless support you people have shown me. In addition, some part of this Ph.D. work was conducted at the Center for Bioproducts and Bioenergy, Washington State University, USA, under the supervision of Birgitte K. Ahring and i would also like to express my warmly gratitude to all my colleagues over there for providing a good working atmosphere and nice company throughout my stay.

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"The universe is full of magical things,

patiently waiting for our wits

to grow sharper."

Eden Phillpotts

THESIS CONTENT

SUMMARY

The negative impacts of fossil fuel consumption on the environment, rising prices for fossil fuels and progressive demand for energy have renewed the interest of society in searching for alternative renewable and sustainable forms of energy. Hence, the alternative energy supply must confirm with some basic requirements including substantial reduction of greenhouse gas emission, strengthening rural and agricultural economies, increasing sustainability of the world transportations system, and capability of being produced from renewable and sustainable sources. Production of biofuels especially bioethanol from lignocellulosic plant biomass seems to be an interesting replacement for conventional fossil fuels. Bioethanol can be applied in many ways; however, today the major use of ethanol is as an oxygenated fuel additive. Blending bioethanol and gasoline has several advantages, like the higher octane number of bioethanol (96-113) increases the octane number of the blended, reducing the need for toxic, octane-enhancing additives. Bioethanol also provides oxygen for the fuel, which will lead to the reduced emission of CO_2 and un-combusted hydrocarbons.

At present, bioethanol is mostly produced from cereals (corn or grain) and sugarcane juice (so called – 1st generation). However, the use of these agricultural crops for bioethanol production is unsustainable in near future scenarios since it is conflicting with food and feed production and perhaps very expensive. Thus, lignocellulosic biomass such as agricultural residues, forestry waste and municipal solid waste present a sustainable source for the production of liquid biofuels and other high valuable biomaterials (2nd generation) because they are abundant and inexpensive. These facts have motivated extensive research toward making an efficient conversion of lignocellulosic materials into sugar monomers for subsequent fermentation to bioproducts.

The complex structure of native lignocellulosic biomass makes it however, difficult for microorganisms to access; it is mainly composed of cellulose, hemicelluloses, and lignin. Therefore, production of liquid biofuels from lignocellulosic biomass creates technical challenges, such as the need for pretreatment to make sugars available for the subsequent fermentation steps.

The main goal of this research was to optimize the wet explosion (WEx) pretreatment process parameters of lignocellulosic biomass for bioethanol production in parallel to a demonstration plant (BornBioFuel) concept. Several lignocellulosic biomasses (Lucerne, *Medicago sativa* (Marshal), ryegrass, *Lolium* (Mathilde), fescue grass, *Festuca arundinacea* (Hykor), cocksfoot grass, *Dactylis glomerata*, (Amba), rye fescue, *Festulolium* (Perun), forage grass - a mixture of 10% red clover grass (Rajah), 10% white clover grass (Klondike), 40% rye fescue (Perun), 20% ryegrass (Indiana), 20% ryegrass (Mikado), and wheat straw) from the island of Bornholm, Denmark, was initially screened for their potential of bioethanol production by employing wet explosion as a modified dilute acid pretreatment and subsequent enzymatic hydrolysis of cellulose fractions.

Wheat straw and cocksfoot grass were identified as the two most promising biomass resources with the highest potential for further optimization of process parameters of the different steps of the whole concept. However, the research focus was finally directed to cocksfoot grass, while wheat straw was used as a reference biomass sample. This is due to the fact that wheat straw has been extensively investigated by many studies dealing with cellulosic bioethanol production especially in Denmark. Wet explosion pretreatment was applied to cocksfoot grass and pretreatment process parameters were optimized using response surface analysis for increasing the production of fermentable sugars. WEx pretreatment temperature (160-210 ^{o}C), retention time (5-20 min) and dilute sulfuric acid (0.2-0.5%) were the investigated process parameters. It was observed that higher pretreatment temperature had major increasing effect on the glucose yield regardless of acid concentration. On the other hand, increasing the acid concentration and process temperature shows a negative effect on the pentose sugars yield together with longer retention time, and facilitated high formation of degradation products. From the overall sugar yields, it was found that higher pretreatment temperature with moderate acid concentration and lower retention time was best for achieving higher total sugars yield. Moreover, when looking at the individual sugar yields from cellulose or hemicellulose fractions, it was obvious that the release of hexose and pentose sugars needs different pretreatment severity, as the conditions where the highest hexose sugars were obtained is detrimental to the release of pentose sugars.

Further pretreatment of cocksfoot grass was carried at high dry matter concentration (25%) together with addition of dilute sulfuric acid or oxygen pressure. The resulting solid fraction was fermented to ethanol by the yeast *Saccharomyces cerevisiae* and liquid hydrolysate was fermented to ethanol by the yeast *Pichia stipitis* CBS 6054 with a higher yields.

This present investigation showed, however, that wet explosion pretreatment of lignocellulosic biomass is an effective pretreatment method that can easily be adapted to the input of different type of biomass materials at high dry matter concentration and can accommodate several types of additives, e.g. (O_2/dilute acid) enabling the production of bioethanol both from hexose and pentose sugars at higher yields.

DANSK SAMMENFATNING

Den stadig stigende negative indflydelse fra afbrænding af fossile brændsler, stigende priser på fossil olie og voksende efterspørgsel efter energi har genskabt en samfundsinteresse for at finde alternative, bæredygtige energiformer. Derfor er det nødvendigt, at alternative energiformer både indebærer en betydelig reduktion af drivhusgasser, og samtidig styrker lokale økonomier og landbrug. Alternativerne bør desuden øge bæredygtigheden af verdens transportsystemer baseret på fornybare ressourcer. Produktion af biobrændstoffer, særlig bioethanol fremstillet på basis af lignocelluloseholdig plantebiomasse, synes at være et interessant alternativ til konventionelle fossile brændsler.

Dette skyldes, at bioethanol kan anvendes på flere måder. I dag er den hyppigste anvendelse som tilsætning til konventionelle brændstoffer, hvor f.eks. tilsætning af bioethanol til benzin øger oktanindholdet og på denne måde overflødiggør brug af giftige, oktanforstærkende kemikalier. Iltindholdet i bioethanol medfører desuden renere forbrænding, som reducerer udledning af både CO_2 og uforbrændte kulbrinter.

I dag fremstilles bioethanol overvejende af majs og saft fra rørsukker (såkaldt 1. generation biobrændstoffer). Imidlertid er anvendelsen af afgrøder til fremstilling af bioethanol ikke bæredygtig, da den er i konflikt med både fødevare- og foderforsyning og desuden er meget dyr. Derimod er lignocelluloseholdig markaffald, affald fra skovbrug samt brændbart affald fra byerne eksempler på bæredygtige ressourcer til produktion af flydende brændstoffer og andre værdifulde biomaterialer (såkaldt 2. generation) fordi de er både talrige og billige. Derfor foregår der omfattende forskning i nedbrydning af lignocelluloser til sukkermonomerer som derefter omdannes til bioprodukter ved fermentering. Mikroorganismer har imidlertid problemer med at omsætte den komplekse struktur af rå, lignocelluloseholdig biomasse, som hovedsagelig består af komponenterne cellulose, hemicellulose og lignin. For at frigøre sukrerne, er det derfor nødvendigt at ubehandlet biomasse udsættes for en forbehandling forud for mikroorganismernes fermentering.

Hovedmålsætningen for nærværende forskningsarbejde var et forbedre vådeksplosion (WEx) forbehandlingen af lignocelluloseholdig biomasse til bioethanolproduktion og benytte resultaterne i et demonstrationsanlæg (BornBioFuel) lokaliseret på Bornholm. Til at begynde med blev en række, forskellige biomasser fra Bornholm undersøgt for deres potentiale i biothanolproduktion ved at benytte en forbehandling som bestod af vådeksplosion og mild syrebehandling, efterfulgt af enzymatisk hydrolyse af cellulosefraktionen. Arterne omfattede: Lucerne (*Medicago sativa*), Rajgræs (Lolium), Svingel (*Festuca arundinacea),* Hundegræs *(Dactylis glomerata),* Rajsvingel

(Festulolium), fodergræs – en blanding af 10% Rødkløver, 10% Hvidkløver, 40% Rajsvingel, 40% Rajgræs (2 sorter) – samt hvedehalm. Hvedehalm og Hundegræs viste sig at være de mest lovende arter med hensyn til optimering af forbehandling og sukkerekstraktion. Til yderligere undersøgelser faldt valget imidlertid på Hundegræs, mens hvedehalm blev benyttet som reference. Baggrunden for dette valg var, at forskning i hvedehalm til bioethanolproduktion allerede var omfattende, i særdeleshed i Danmark.

For at maksimere produktion af mikrobiel omsættelige sukrer, blev Hundegræs udsat for forbehandling med vådeksplosion, og øvrige processparametre blev optimeret vha. "Response Surface Analysis". Processparametrene var følgende: WEx temperatur (160-210°C), retentionstid (5-20 min.) og syrebehandling blev gennemført med 0,2-0,5% svovlsyre. Det blev konstateret, at jo højere forbehandlingstemperatur desto højere glukoseudbytte; uanset syrekoncentrationen. Derimod havde høj syrekoncentration, høj temperatur og lang retentionstid en negativ indflydelse på sukkerudbyttet mht. pentosesukrer, og der blev i stedet dannet mange nedbrydningsprodukter som hæmmer fermentering. Generelt var høj temperatur i kombination med moderat syrekoncentration og kort retentionstid, den mest effekive metode til at opnå et højt udbytte af sukrer. Dog viste det sig ved nærmere undersøgelse, at forbehandling som var optimal for ekstraktion af hexosesukrer var skadelig for ekstraktion af pentosesukrer.

Yderligere forbehandling af Hundegræs blev foretaget ved tilsætning af fortyndet svovlsyre eller ved iltbehandling i forbehandlinger med højt tørstofindhold (25%). Resultatet blev hhv. en tør og en vandig fraktion. Den tørre blev fermenteret til ethanol af bagegær *Saccharomyces cerevisae* og den vandige fraktion blev fermenteret med et højere udbytte af gæren *Pichia stiptis* CBS6054.

Nærværende studier viste, at forbehandling af lignocelluloseholdig biomasse vha. vådeksplosion er effektiv da metoden kan behandle en række forskellige biomasser med højt tørstofindhold. Desuden rummer metoden mulighed for at justere processen med både ilt-og syrebehandling som muliggør produktion af bioethanol med højt udbytte på grundlag af både pentose- og hexosesukrer.

INTRODUCTION AND AIM OF THE PhD STUDY

Nowadays both energy crisis and climate change are key issues all over the world. There will be severe energy shortage in the coming 50 years. According to current research and future predictions, the crude oil will run out within 40 to 70 years, and natural gas will be finished within 50 years (Courtney and Dorman, 2003). Global average temperature is predicted to increase 1.4 to 5.8 °C by year 2100 and continue to rise long after that (Dow and Downing, 2006). Several investigations point out that this will inevitably lead to drought, flooding, increases in hurricanes and tornadoes and possibly widespread crop failures (Sen, 2009; Mills, 2009). Global warming as the result of climate change is an established fact. It is now widely accepted that it is caused by the rapidly increasing concentrations of greenhouse gas (CO_2 and others) in the atmosphere, which is emitted mainly by the combustion of fossil fuels containing carbon like coal, oil, and natural gas (Jaynes, 2010). Security of energy supply, especially sustainable energy, and reduction of CO_2 emission are priorities on agenda worldwide. Renewable energy is politically demanded. The European Community has agreed targets for 2020 on renewable energy, which established a high standard for all Member States, aiming a 20% share of renewable energy sources by the year 2020 and a 10% share of renewable energy specifically in the transport sector (European Commission Energy, 2010).

The use of renewable biomass resources to produce liquid biofuels such as bioethanol offer attractive solutions to reducing greenhouse gas (GHG) emissions, decreasing reliance on foreign oils, addressing energy security concerns, strengthening rural and agricultural economies, and increasing sustainability of the world transportations system (Demirbas, 2007). Apart from biofuels, many other valuable products for chemical and pharmaceutical industry can be produced from organic byproducts through microbial fermentation (Thomsen, 2005). Most current bioethanol production processes (1^{st} generation) utilize more easily degradable biomass feedstocks such as cereals (corn or grain) and sugarcane juice. However, the utilization of these agricultural crops exclusively for energy production is heavily conflicting with food and feed production (Pimental et al., 2009; Wheals et al.,

1999). Great effort is enforced on advancing a cellulosic bioethanol concept (2nd generation) that utilizers lignocellulosic biomass.

Lignocellulosic plant biomass such as agricultural waste (wheat straw, corn stalks, and sugarcane bagasse), industrial waste (pulp and paper), and municipal solid waste and forestry residues are promising resources because they are the most abundant and inexpensive natural renewable organic material that exist on earth (Zaldivar et al., 2001). As most often being a byproduct from food and feed production, lignocellulosic biomass does not compete with the production of edible crops (Chen and Qiu, 2010 and Petersson et al., 2007) and has the potential to be the feedstock for the production of a considerable proportion of transport fuels if cost effective conversion processes are available (Kristensen et al., 2008). From the economical point of view, lignocellulose has an advantage over other agriculturally important biofuels feedstocks such as cornstarch, potatoes and sugarcane juice because it can be easily produced at significantly lower cost than food crops. The economically viable utilization of lignocellulosic plant biomass for the production of biofuels and other biobased products is, however, still a challenge. The lignocellulosic biomass structure is highly complex, mainly composed of cellulose, hemicelluloses, and lignin that are not directly accessible for microbial degradation (Chen and Qiu, 2010). Therefore, pretreatment is a key process to break the lignocellulosic structure and to make it available for hydrolyzing enzymes to release the sugar monomers that can finally be converted into ethanol or any other valuable bioproducts (Georgieva et al., 2008).

The goal of pretreatment is to increase the biomass surface area and break the lignin seal in order to release cellulose and hemicellulose, and decrease the crystallinity of the cellulose. Pretreatment is among the most costly steps in biochemical conversion of lignocellulosic biomass, accounting for up to 40% of the total processing cost (Eggeman and Elander, 2005; Wyman et al., 2005; Lynd, 1996; Percival Zhang et al., 2009). Thus, cost-effective pretreatment of lignocellulosic biomass is a major challenge of cellulosic biofuels and bioproducts technology research and development in recent years (Hamelinck et al., 2005). The choice of a suitable pretreatment method and the adjustment of the

pretreatment parameters are crucial for the efficiency of the subsequent conversion of any biomass in a biorefinery concept.

Various pretreatment methods have been proposed to make lignocellulosic biomass susceptible to enzymatic and microbial conversion (Galbe and Zacchi, 2002; Hendriks and Zeeman, 2009), wet oxidation (Schmidt and Thomsen, 1998), dilute acid hydrolysis (Saha et al., 2005), and steam explosion (Ballesteros et al., 2006). Wet oxidation pretreatment method has been applied in laboratory and pilot-scale process for fractionation of several lignocellulosic biomass such as wheat straw, sugarcane bagasse, softwood, corn stover, clover-ryegrass mixtures and rice husk (Ahring and Westermann, 2007; Westermann and Ahring, 2005; Varga et al., 2003; Martin et al., 2008; Banerjee et al., 2009; Palonen et al., 2004). Wet oxidation make use of oxidizing agent such as H_2O_2, water and air or oxygen at pretreatment temperatures around 180-200^{o}C for 5-30 min (McGinnis, 1983). Dilute acid pretreatment is widely recognized in the field of biofuels production for pretreatment of lignocellulosic biomass. High yields of sugars from lignocellulosic biomass can be achieved from dilute acid pretreatment. The mode of action is hemicellulose removal and thus, enhances the digestibility of cellulose in the residual solids. The process is mainly carried out under high temperature and low acid concentration and the most widely used acid under this process is sulfuric acid (Kim et al., 2011; Saha and Bothast, 1999; Foston and Ragauskas, 2010). Steam explosion is the most commonly used method for pretreatment of lignocellulosic biomass because it does not require the addition of chemicals (uncatalyzed steam explosion). The mode of action involves treatment of chipped biomass with high-pressure saturated steam, followed by a sudden drop in pressure, which makes the materials undergo an explosive decompression. This sudden pressure release reduces the temperature and quenches the reaction at the end of the pretreatment. Steam explosion is typically initiated at temperatures around 160-260^{o}C (corresponding pressure, 0.69-4.83 MPa) for several seconds to a few minutes before the material is exposed to atmospheric pressure (Boussaid et al., 1999; Kurabi et al., 2005; Varge et al., 2004; Ruiz et al., 2006; Carrasco et al., 1994; Josefsson et al., 2002; Ballesteros et al., 2004; Mes-Hartree and Saddler, 1983; Sun et al., 2004; Laser et al., 2002; Cullis et al., 2004).

The focus of the present PhD thesis was the optimization of key processes of an integrated concept for cost efficient cellulosic biofuels production that was the basis for a projected demonstration plant (BornBioFuel concept). The concept integrates biomass pretreatment, enzymatic hydrolysis of the cellulose, fermentation of both C6 and C5 monomers and the conversion of residual organic matter into valuable products. Residual salts are separated to be utilized as organic fertilizer while the residual water stream is recirculated as process water (see Fig. 1).

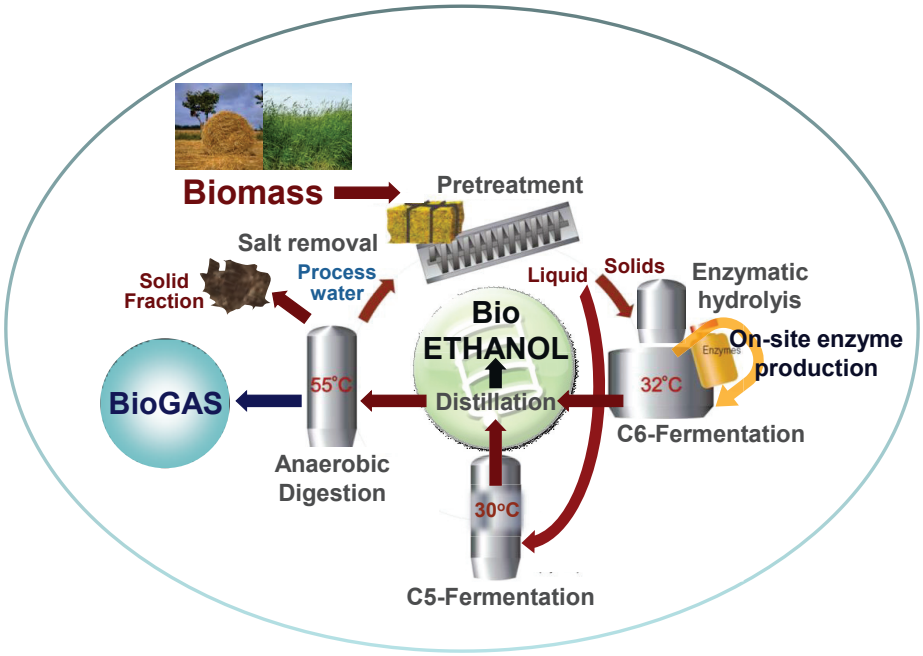

Fig. 1. The BornBioFuel concept – basis for the optimization of different steps of cellulosic biofuels processes.

The optimization of the concept included the most efficient combination of pretreatment, solid-liquid separation, enzymatic hydrolysis of sugar polymers, fermentation of both C6 and C5 sugar monomers and the separation of residual organic matter for further processing. The key processes for the PhD were to adapt the concept to potential types of

biomass other than wheat straw, to tailor the pretreatment method for a highly efficient C6 and C5 sugar fermentation into ethanol with the target to reduce the costs of cellulosic ethanol production significantly.

The PhD thesis consists of four parts: (Review paper – paper I) the general overview of biorefinery concepts with existing technologies for biofuels production and applications in parallel to the BornBioFuel concept; the second part (Paper II-III) presents the original contribution of the thesis with an insight into different biomass resources from the island of Bornholm, Denmark for their potential of bioethanol production and the identification of the most promising types of biomass which can be used for the BornBioFuel plant; the third part (Paper IV) presents the pretreatment at high dry matter concentration of w/w 25% with addition of oxygen or acid and fermentation of hexose sugars (C6) to ethanol by the yeast *Saccharomyces cerevisiae*, with quantifying the effect of wet explosion process parameters on the ethanol yield; the fourth part (Paper V): presents the possibility of complete utilization of the hemicellulose hydrolysate to ethanol by the yeast *Pichia stipitis*. The effect of potential inhibitors produced during the pretreatment on the *Pichia* fermentation was investigated.

REFERENCES

Ahring, B. K., Westermann, P., 2007. Coproduction of bioethanol with other biofuels. Adv. Biochem. Eng. Biotechnol. 108, 289-302.

Ballesteros, I., Negro, M. J., Oliva, J. M., Cabanas, A., and Ballesteros, M., 2006. Ethanol produced from steam-explosion pretreated wheat straw. Appl. Biochem. Biotechnol. 129-132, 496-508.

Ballesteros, M., Oliva, J. M., Negro, M. J., Manzanares, P., Ballesteros, I., 2004. Ethanol from lignocellulosic materials by a simultaneous saccharification and fermentation process (SFS) with *Kluyveromyces marxianus* CECT 10875. Process Biochem. 39, 1843-1848.

Banerjee, S., Sen, R., Pandey, R. A., Chakrabarti, T., Satpute, D., Giri, B. S., Mudliar, S., 2009. Evaluation of wet air oxidation as a pretreatment strategy for bioethanol

production from rice husk and process optimization. Biomass and Bioenergy 33, 1680-1686.

Boussaid, A., Robinson, J., Cai, Y. J., Gregg, D. J., Saddler, J. R., 1999. Fermentability of the hemicellulose-derived sugars from steam-explosion softwood (Douglas fir). Biotechnol. Bioeng. 64, 284-289.

Carrasco, J. E., Saiz, M. C., Navarro, A., Soriano, P., Saez, F., Martinez, J. M., 1994. Effects of dilute acid and steam explosion pretreatments on the cellulose structure and kinetics of cellulosic fraction hydrolysis by dilute acids in lignocellulosic materials. Appl. Biochem. Biotechnol. 45, 23-34.

Chen, H., Qiu, W., 2010. Key technologies for bioethanol production from lignocellulose. Biotechnol. Advances 28, 556-562.

Courtney, B., Dorman, D., 2003. World Wide Fossil Fuels. Chemistry Department of Louisiana State University.

Cullis, I. F., Saddler, J. N., Mansfield, S. D., 2004. Effect of initial moisture content and chip size on the bioconversion efficiency of softwood lignocellulosics. Biotechnol. Bioeng. 85, 413-421.

Demirbas, A., 2007. Progress and recent trends in biofuels. Progress in Energy and Combustion Science 33, 1-18.

Dow, K., Downing, T., 2006. The Atlas of Climate Change: Mapping The World's Greatest Challenge. Los Angeles: University of California Press.

Eggeman, T., Elander, R. T., 2005. Process and economic analysis of pretreatment technologies. Bioresource Technology 96, 2019-2025.

European Commission Energy, 2010. Targets. Renewable Energy: http://ec.europa.eu/energy/renewables/targets_en.htm. Retrieved 23 August 2012.

Foston, M., Ragauskas, A. J., 2010. Changes in the structure of the cellulose fiber wall during dilute acid pretreatment in *populus* studied by ^{1}H and ^{2}H NMR. Energy & Fuels 24, 5677-5685.

Galbe, M., Zacchi, G., 2002. A review of the production of ethanol from softwood. Appl. Microbiol. Biotechnol. 59, 618-628.

Georgieva, T. I., Hou, X., Hilstrøm, T., Ahring, B. K., 2008. Enzymatic hydrolysis and ethanol fermentation of high dry matter wet-exploded wheat straw at low enzyme loading. Appl. Biochem. Biotechnol. 148, 35-44.

Hamelinck, C. N., Hooijdonk, V. G., Faaij, A. P. C., 2005. Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle-and long term. Biomass and Bioenergy 28, 384-410.

Hendriks, A. T. W. M., Zeeman, G., 2009. Pretreatment to enhance the digestibility of lignocellulosic biomass. Bioresource Technology 100, 10-18.

Jaynes, D., 2010. Global Warming: Myths & Realities. Social Sciences, SOC 461-11.

Josefsson, T., Lennholm, H., Gellerstedt, G., 2002. Steam explosion of aspen wood. Characterisation of reaction products. Holzforschung 56, 289-297.

Kamm, B., Kamm, M., 2004b. Biorefinery – systems. Chem. Biochem. Eng. Q. 18, 1-6.

Kim, S, B., Lee, J. H., Oh, K. K., Lee, S. J., Lee, J. Y., Kim, J. S., Kim, S. W., 2011. Dilute acid pretreatment of barley straw and its saccharification and fermentation. Biotechnol. Bioprocess Eng. 16, 725-732.

Kristensen, J. B., Thygesen, L. G., Felby, C., Jørgensen, H., and Elder, T., 2008. Cell-wall structure changes in wheat straw pretreated for bioethanol production. Biotechnol. for Biofuels 1, 1-9.

Kurabi, A., Berlin, A., Gilkes, N., Kilburn, D., Bura, R., Robinson, J., Markov, A., Skomarovsky, A., Gusakov, A., Okunev, O., Sinitsyn, A., Gregg, D., Xie, D., Saddler, J., 2005. Enzymatic hydrolysis of steam-exploded and ethanol organosolv-pretreated Douglas-Firby novel and commercial fungal cellulases. Appl. Biochem. Biotechnol. 121, 219-230.

Laser, M., Schulman, D., Allen, S. G., Lichwa, J., Antal Jr., M. J., Lynd, L. R., 2002. A comparison of liquid hot water and steam pretreatments of sugar cane bagasse for bioconversion to ethanol. Bioresource Technology 81, 33-44.

Lynd, L. R., 1996. Overview and evaluation of fuel ethanol from cellulosic biomass: Technology, Economics, the Environment, and Policy. Annu. Rev. Energy Environ. 21, 404-465.

Martin, C., Thomsen, M. H., Hauggaard-Nielsen, H., Thomsen, A. B., 2008. Wet oxidation pretreatment, enzymatic hydrolysis and simultaneous saccharification and fermentation of clover-ryegrass mixtures. Bioresource Technology 99, 8777-8782.

McGinnis, G. D., Wilson, W. W., Mullen, C. E., 1983. Biomass pretreatment with water and high-pressure oxygen. The wet-oxidation process. Ind. Eng. Chem. Prod. Res. Dev. 22, 352-357.

Mes-Hartree, M., Saddler, J. N., 1983. The nature of inhibitory materials present in pretreated lignocellulosic substrates which inhibit the enzymatic hydrolysis of cellulose. Biotechnol. Lett. 5, 531-536.

Mills, D. M. MA., 2009. Climate Change, Extreme Weather Events, and US Health Impacts: What Can We Say? Jour. of Occupational and Environ. Medicine 51, 26-32.

Palonen, H., Thomsen, A. B., Tenkanen, M., Schmidt, A. S., Viikari, L., 2004. Evaluation of wet oxidation pretreatment for enzymatic hydrolysis of softwood. Appl. Biochem. Biotechnol. 117, 1-17.

Percival Zhang, Y. H., Berson, E., Sarkanen, S., Dale, B.E. 2009. Pretreatment and biomass recalcitrance: Fundamentals and Progress. Appl. Biochem. Biotechnol. 153, 80-83.

Petersson, A., Thomsen, M. H., Hauggaard-Nielsen, H., Thomsen, A. B., 2007. Potential bioethanol and biogas production using lignocellulosic biomass from winter rye, oilseed rape and faba bean. Biomass and Bioenergy 31, 812-819.

Pimental, D., Marklein, A., Toth, M. A., Karpoff, M. N., Paul, G. S., McCormack, R., Kyriazis, J., Krueger, T., 2009. Hum. Ecol. 37, 1-12.

Ruiz, E., Cara, C., Ballesteros, M., Manzanares, P., Ballesteros, I., Castro, E., 2006. Ethanol production from pretreated olive tree wood and sunflower stalks by an SSF process. Appl. Biochem. Biotechnol. 129, 631-643.

Saha, B. C., Iten, L. B., Cotta, M. A., Wu, Y. V., 2005. Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. Process Biochem. 40, 3693-3700.

Saha, B C., Bothast, R. J., 1999. Pretreatment and enzymatic saccharification of corn fiber. Appl. Biochem. Biotechnol. 76, 65-77.

Schmidt, A. S., Thomsen, A. B., 1998. Optimization of wet oxidation pretreatment of wheat straw. Bioresource Technology 64, 139-151.

Sen, Z., 2009. Global waeming threat on water resources and environment: a review. Environ. Geology 57, 321-329.

Sun, X. F., Xu, F., Sun, R. C., Wang, Y. X., Fowler, P., Baird, M. S., 2004. Characteristics of degraded lignins obtained from steam exploded wheat straw. Polym. Degrad. Stabil. 86, 245-256.

Thomsen, M. H., 2005. Complex media from processing of agricultural crops for microbial fermentation. Appl. Microbiol. Biotechnol. 68, 598-606.

Varga, E., Reczey, K., Zacchi, G., 2004. Optimization of steam pretreatment of corn stover to enhance enzymatic digestibility. Appl. Biochem. Biotechnol. 113, 509-523.

Varga, E., Schmidt, A. S., Reczey, K., Thomsen, A. B., 2003. Pretreatment of corn stover using Wet Oxidation to enhance enzymatic digestibility. Appl. Biochem. and Biotechnol. 104, 0273-2289.

Westermann, P., Ahring, B. K., 2005. The biorefinery for production of multiple biofuels. In: Lens, P., Westermann, P., Haberbauer, M., Moreno, A., editors. Biofuels for fuels cells: Renewable energy from biomass fermentation. London, UK: IWA Publishing, p 194-205.

Wheals, A. E., Basso, L. C., Alves, D. M. G., Amorim, H. V., 1999. Fuel ethanol after 25 years. Trends Biotechnol. 17, 482-487.

Wyman, C. E., Dale, B. E., Elander, R. T., Holtzapple, M., Ladisch, M. R., Lee, Y. Y., 2005b. Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover. Bioresource Technology 96, 2026-2032.

Zaldivar, J., Nielsen, J., Olsson, L., 2001. Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. Appl. Microbiol. Biotechnol. 56, 17-34.

Review paper

Biofuels and biomaterials production in a biorefinery – key features for sustainable and economically viable concepts

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Biofuels and biomaterials production in a biorefinery – key features for sustainable and economically viable concepts

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Abstract

Depleting fossil fuels and the increasing demand for energy has necessitated the move to alternative renewable forms of energy. Lignocellulosic biomass presents a sustainable and renewable source for the production of high valuable biobased products in a biorefinery system. This current paper reviews the concept of biorefinery system in relation with different process steps in conversion of lignocellulosic biomass. Biorefinery similar to petroleum refinery has the capabilities to convert several types of biomass into a wide range of bioproducts such as energy, fuels, chemicals, food, feed, and etc. through jointly applied conversion technologies. However, there still the needs for the development and implementation of integrated biorefinery system which can significantly handle all the fractions of biomass to produce varieties of products on single platform at a continuous process system in a diversify scenario. Such a system should be able to utilize different kinds of technologies for conversion of the raw materials and be flexible in processing which will greatly reduce market and technical risk, and extensively lower the process costs. Therefore, the main focus should be directed towards the optimization and validation of different process steps involves in biorefinery system. In this way, the cost of production of energy and fuels as the driving force for the development of biorefinery as well as high-value chemicals will be significantly reduced to be more cost-competitive with fossil fuels for their commercial scaling-up.

Keywords: Biorefinery concept; biomass plants; lignocellulosic feedstock; biofuels & bioproducts; pretreatment; development & applications.

INTRODUCTION

The global energy supply is dominated by fossil fuels (petroleum, natural gas, coal, minerals). Currently, fossil energy resources account for about 79% of the global energy consumption (European Commission, 2005) and its use is unsustainable at current and future rates consumption. The combustion of the above-mentioned fossil fuels also contributes significantly to environmental pollution and greenhouse gas (GHG) accumulation on the biosphere. Furthermore, the depletion of fossil resources and the increasing demand for energy has necessitated the search for alternative renewable forms of energy. Biomass (organic materials of biological origin) present a sustainable and renewable source for the production of multiple products, including higher-value chemicals for industrial purposes, as well as liquid fuels for transportation sector and power (Sauer et al., 2008).

The use of renewable biomass resources to produce liquid biofuels such as bioethanol offer attractive solutions to reducing GHG emissions, decreasing reliance on foreign oils, addressing energy security concerns, strengthening rural and agricultural economies, and increasing sustainability of the world transportations system (Demirbas, 2007). Biomass can be sustainably utilized for the production of clean and environmental-friendly energy and biobased products in several ways. One main approach that has gained much attention in the recent years is by integrated processing in biorefinery system (Holm-Nielsen et al., 2007). The term “biorefinery” was initially established by National Renewable Energy Laboratory (NREL) during 1990, for the utilization of biomass for production of fuels and other biobased products. This term refers to a facility for achieving large-scale integrated production of fuels, power, chemicals, food, and feed from biomass (fig. 1). It is analogous to petroleum refinery producing a wide variety of products from crude oil, the same principles can also be applied to a biomass based refinery (biorefinery) to produce a sustainable and clean biobased products (Kamm et al., 2006).

A wide range of products is delivered with multiple end uses, including: low-volume and high-value specialty chemicals that have niche uses in the food and other industries; high-volume and low-value liquid fuels for wide-spread use in the transport industry; heat,

electricity, and etc. (Fernando et al., 2006). The diversity in products gives a high degree of flexibility to changing market demands and allows such a system many options for gaining revenues and maintaining high profitability (Lasure and Zhang, 2004). This paper conveys the different concept in biorefinery systems and the integrated production process scheme of valuable biobased products. The overview of various process steps of the processing of lignocellulosic biomass in biorefinery systems are presented and discussed. The raw materials for biorefinery processes and applications and use of its various end-products were reviewed to a large extend.

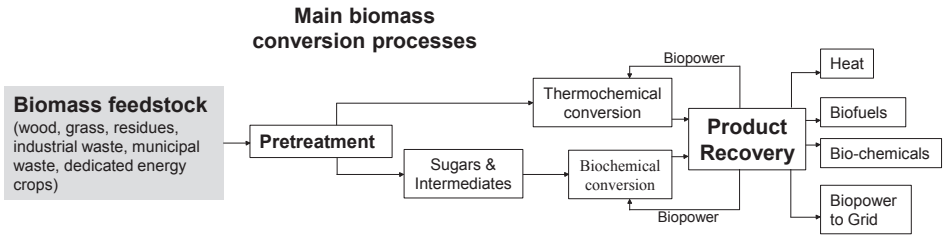

Fig. 1. Overview of an integrated biorefinery system, similar to petroleum refinery producing a wide range of high valuable biobased products from biomass feedstock.

Biorefinery systems

Basic principles of biorefinery system

The first generation biofuels concept are the current biofuels produced primarily from agricultural crops such as corn ethanol in US, sugarcane ethanol in Brazil, palm oil biodiesel in Malaysia, and oilseed rape biodiesel in Germany (Sims et al., 2008). The technologies for first generation biofuels are matured to its commercial markets phase. The technology utilizes more easily degradable biomass feedstocks such as cereals (corn or grain) and sugarcane juice as input raw materials producing fixed amount or wide variety of bioproducts including ethanol and distillers dried grains with protein (DDG) as an animal feed (Bothast and Schlicher, 2005). However, the utilization of these agricultural crops exclusively for energy production is heavily conflicting with food and feed production (Pimental et al., 2009; Wheals et al., 1999), which sets limits in the increasing production

of biofuels of 1st generation. But, as biofuels offer a potentially attractive solution to reducing the use of fossil fuels, the demand for first generation biofuels will continue to grow especially, sugarcane ethanol that will continue to be produced sustainable in many countries around the world. Great effort is enforced on advancing a biorefinery technology, called the "lignocellulose feedstock (LCF) refinery" – 2nd generation biofuels concept (Kamm and Kamm, 2007), a system which is further characterized as "the carbon slaughterhouse" as described by Ahring and Langvad, (2008). It allows a mix of input lignocellulosic biomass, has the ability to use various types of processing technologies that can process almost 100% of input raw materials, and has the capability to produce a mix of higher-value chemicals while coproducing biofuels (Fernando et al., 2006). But the process technology of this system is complex and still in research and development phase with quite number of commercial, demonstration and pilot-scale plants around the world. Therefore, lignocellulosic materials can offer the potential to provide novel biofuels in the coming years. Hence, the need for research and development of this technology is essential in order to meet the demand of ever dream greener society and biobased products. Following, commercialization and policy support is needed for current and near-term opportunities to quickly grow the lignocellulosic biorefinery industry from its present phase.

Like petroleum, lignocellulosic biomass has a complex composition. The goal of petroleum-based refinery is separation of main fractions of the substrate and its processing in order to obtain wide range of simple to handle and well defined chemically pure products from hydrocarbons. This principle of petroleum refineries must be transferred to biorefineries in order to create a healthier and efficient working biobased refinery (Kamm et al., 2006). The renewable and non-renewable refinery concepts are presented in Figure 2.

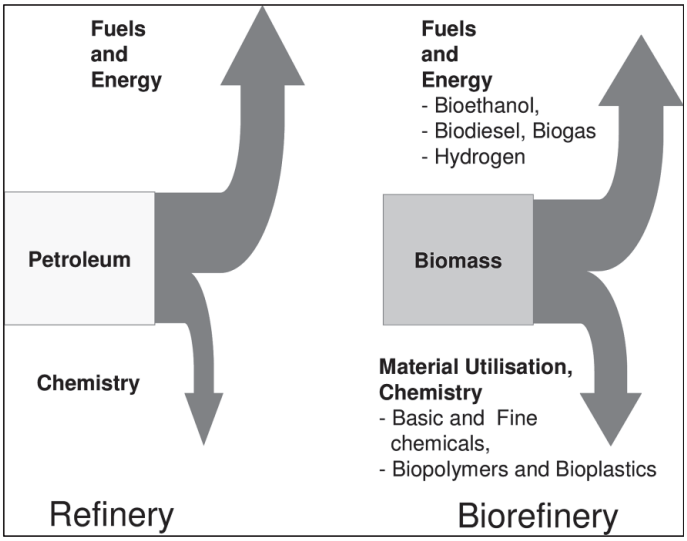

Fig. 2. Comparison of the basic principles of conventional refinery and biorefinery (Kamm et al., 2006).

Choice of raw materials for biorefinery processes

The choice of raw materials for biorefinery processes largely depends on its cost and availability, and it can also incorporate the conversion process cost. It is well documented that lignocellulosic biomass has great potentials over agricultural crops because it is available at large quantities, it is cheap and it has no controversy cause over feed and food production. Claassen et al. (1999) reported that the costs of agricultural crops such as cereals (corn and grain) usually account for 40-70% of the production costs of ethanol, and thus, the competitive production of ethanol on the basis of these materials is not realistic in near-future. On the other hand, lignocellulosic biomass has more complex structure; and therefore, effective utilization of all the components to produce high-value biobased products would play a significant role in economic viability process.

Lignocellulosic plant biomass such as agricultural waste (wheat straw, corn stalks, and sugarcane bagasse), industrial waste (pulp and paper), and municipal solid waste and forestry residues are a promising resources because they are the most abundant and

inexpensive natural renewable organic material that exist on earth (Zaldivar et al., 2001). As most often being a byproduct from food and feed production, lignocellulosic biomass does not compete with the production of edible crops (Chen and Qiu, 2010 and Petersson et al., 2007) and has the potential to be the feedstock for the production of a considerable proportion of transport fuels if cost effective conversion processes are available (Kristensen et al., 2008). From the economical point of view, lignocellulose has an advantage over other agriculturally important biofuels feedstocks such as cornstarch, potatoes and sugarcane juice because it can be easily produced at significantly lower cost than food crops. The utilization of lignocellulosic plant biomass for production of biofuels and other biobased products is a challenge for economical viable because there are a number of technical barriers that need to be overcome before their potential can be realized. The lignocellulosic biomass structure is highly complex, mainly composed of cellulose, hemicelluloses, and lignin that are not directly accessible for microbial degradation (Chen and Qiu, 2010). Therefore, pretreatment is normally needed to break the lignocellulosic structure and to make it available for hydrolyzing enzymes to release the sugar monomers that can finally be converted into ethanol or any other valuable products (Georgieva et al., 2008).

Different types of biorefinery system

Phase I biorefinery

The biorefinery system has been categorized into three different types (Kamm et al., 2006; van Dyne et al., 1999; Fernando et al., 2006). The Phase I biorefinery plant has fixed processing capabilities and uses grain as an input feedstock. A dry mill ethanol plant is an example of phase I biorefinery which produces a fixed amount of ethanol, feed co-products, and carbon dioxide (Fernando et al., 2006). It has almost no flexibility in processing and, thus, this type of plant can be used for comparable purposes only (Kamm et al., 2006).

Phase II biorefinery

A Phase II biorefinery is the current wet milling technology that uses grain feedstock as input materials similar to dry milling. Contrary to Phase I, it has the capability to produce a wide variety of end products depending on product demand, market prices, and contract obligations. These products can includes but not limited to starch, high-fructose corn syrup, ethanol, corn oil, corn gluten feed and meal. This type of plant shares the opportunity to integrate industrial product lines with existing agricultural production units (Kamm et al., 2006).

Phase III biorefinery

Phase III biorefinery is the most advanced biorefinery that uses a combination of biomass feedstock to produce multiple products by integration of various technologies. It has the ability to use various types of processing technologies and has the capability to produce a mix of higher-value chemicals while coproducing ethanol, which is mainly based on high-value low-volume and low-value high-volume principles (Fernando et al., 2006). However, the so-called Phase III biorefineries are mainly four complex biorefinery systems (whole-crop, green, biorefinery two platforms concept – the sugar platform and the syngas platform, and lignocellulose feedstock – LCF) and they are still in research and developing phase (Kamm and Kamm, 2004b). The four complex phase III biorefinery systems, which will be discussed in more detail in this chapter, are listed below:

- Whole-crop biorefinery
- Green Biorefinry
- Two platform concept biorefinery
- Lignocellulose feedstock (LCF) biorefinery

Whole-crop biorefinery: The input raw materials for the whole-crop biorefinery are cereals, such as wheat, rye, maize. The starting point of the process is the mechanical separation of material into straw and grains, and then each fraction can be utilized in different unit operations. The straw fractions, such as chaff, nodes, leaves, and stems can be further processed as a lignocellulosic feedstock in a lignocellulose feedstock biorefinery system (LCF). Besides, there is possibility of separation of straw into cellulose, hemicellulose, and

lignin and further processed them in a separate product lines. The straw is the main input raw material for the production of wide range of chemicals, fuels and pharmaceutical products. It can also be subjected to pyrolysis process with the aim of producing syngas where syngas is the basic material for synthesis of fuels and methanol. The grains can be easily converted into starch or used directly after grinding to meal, fructose, syrup, and fuels or feed (Kamm and Kamm, 2007).

Green biorefinery: A green biorefinery is said to be a multi-product system which handle their refinery cuts, fractions and products in accordance with the physiology of the corresponding plant material, which is maintenance and utilization of diversity of syntheses achieved by nature (Kamm and Kamm, 2004a). A green biorefinery uses natural wet biomass generated from untreated products, e.g. grass, clover, alfalfa or immature cereal. As they are mostly herbaceous crops, they contain relatively low percent of lignin, which makes their carbohydrate fractions more accessible for processing in green plants. The green biomass substrate is firstly treated in their natural form by wet-fractionation to produce a fiber-rich press cake (solid fraction) and a nutrient-rich green juice (liquid fraction). The solid fraction contains cellulose, valuable dyes and pigments, crude drugs, and other organics. The solid fraction can be also used for the production of green feed pellets, as a raw material for the production of chemicals, such as levulinic acid, and for conversion to syngas and synthetic biofuels. The liquid fraction is a basis for production of proteins, free amino acids, organic acids, dyes, enzymes, hormones, other organic substances, and minerals (Fernando et al., 2006).

Two-platform concept biorefinery: The two platform concept is made of two steps of biomass conversion to gain valuable end products (fig. 3), where biomass rich in carbohydrates on average of 75 percent can be standardized over a “intermediate sugar platform”, as a basis for further conversion, but it can also be converted thermochemically into synthesis gas (Kamm et al., 2006). The “sugar platform” is based on biochemical conversion processes and mainly focuses on fermentation of sugars extracted from the biomass. The “syngas platform” is based on thermochemical conversion processes with application of gasification of biomass feedstocks and by-products. Other technologies are being used under this process other than biomass gasification, which include

hydrothermolysis, pyrolysis, thermolysis, and combustion. These processes mainly focus only on the utilization of carbohydrates content of the biomass together with the available carbon and hydrogen of the biomass. Other component of the biomass, such as proteins, lignin, oils, amino acids, and fats are not utilized (Kamm and Kamm, 2007). The main advantage of this complex system is the low-tech technology used in biomass conversion with the aim of producing a wide variety of biobased products.

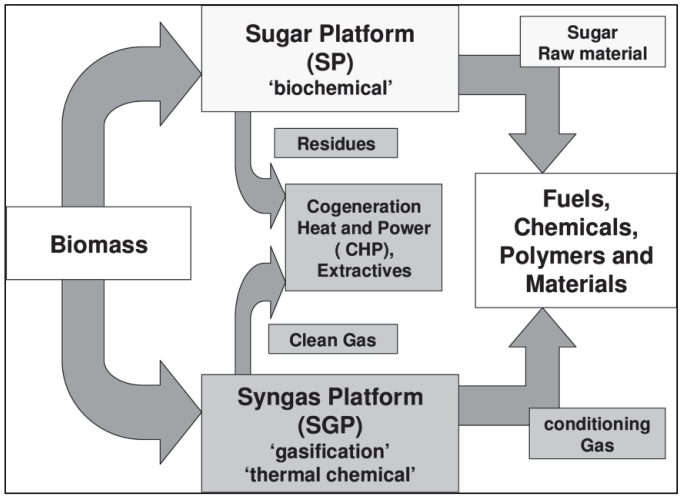

Fig. 3. Two platform biorefinery concept (Kamm et al., 2006).

Lignocellulose feedstock (*LCF*) *biorefinery*: The lignocellulose feedstock biorefinery operates on naturally dry raw material, which contains three chemical fractions, cellulose, hemicellulose, and lignin. The cellulose and hemicellulose can be converted to their sugar fractions through the hydrolysis process (Van Dyne et al., 1999). LCF biorefinery system has advantage over the above-mentioned biorefinery systems because of the availability and low cost of its raw materials, it also has the ability to utilize varieties of feedstock (lignocellulosic biomass). However, there is still the need for further development and optimization of this technology in order fully commercialize its products, for example, in the area of lignocellulose fractionation into cellulose, hemicellulose, and lignin at the same

time, to utilize lignin in the chemical industry for production of phenolic compounds (Kamm and Kamm, 2007).

The LCF biorefinery uses biomass feedstocks with complex structures, such as straw, reed, grass, wood, paper-waste, and municipal solid waste as an input raw material to generated wide range of renewable and sustainable biobased products. The basic chemical reactions of lignocellulose conversions that occur in a LCF biorefinery are shown below (Kamm et al., 2006).

$$\text{Lignocellulose} + H_2O \rightarrow \text{Lignin} + \text{Cellulose} + \text{Hemicellulose}$$
$$\text{Hemicellulose} + H_2O \rightarrow \text{Xylose}$$
$$\text{Xylose } (C_5H_{10}O_5) + \text{acid Catalyst} \rightarrow \text{Furfural } (C_5H_4O_2) + 3H_20$$
$$\text{Cellulose}(C_6H_{10}O_5) + H_2O \rightarrow \text{Glucose } (C_6H_{12}O_6)$$

Biofuels, chemical solvents, polymers, adhesives and etc. are the basic products from lignocellulose. Hemicellulose sugars are vital part of lignocellulose because it can be converted to furfural which is one of the starting points for synthesis of numerous products, which includes Nylon 6 and Nylon 6.6 that are currently produced from petroleum based material because its market size is huge (Van Dyne et al., 1999). Additionally, furfural has many applications: it can be applied in the refining of motor oils and as cleaning agents in liquid fuels. The hydrolysis of cellulose to glucose in order to produce varieties of valuable biobased products, such as organic acids, biofuels, and other fermentation products can be carried out via enzymatic processing or chemical processing. On the other hand, the utilization of lignin fractions as an adhesive, or binder and a fuel for direct combustion is limited when compare with cellulose and hemicellulose fractions (Fernando et al., 2006).

A crucial step in making this system a healthier working biobased refinery is to establish integrated production processes capable of efficiently converting a broad range of lignocellulosic biomass into affordable high-value products such as pharma, food and feed, bioplastics and polymers, bulk chemicals, and biofuels, biopower, and heat. As an integrated biorefinery, it should employ novel technologies and diverse lignocellulosic biomass which will need significant investments in research, development, and deployment projects to reduce costs and thus, improve competitiveness with fossil products. Furthermore, it must also optimize the use of raw materials (lignocellulosic biomass) to

create a product mix that can match to market demand and compete with the current products from fossil oil.

Those requirements can be achieved by applying the principles of diversity, as it's known from studying natural ecosystems that a diversity of organisms brings stability to the ecosystem. When a system is dominated by a single crop, organism or species, the whole structure becomes vulnerable to complete collapse. Such scenarios can be avoided if high value multiple products are targeted in biorefinery systems and when all the parts of biomass is fully converted to products. This means that any byproducts generated in one part of biorefinery can serve as substrates in another, for example, when integrating the production of liquid biofuels with biogas. The residual organic matter in the effluent from ethanol fermentation process can be converted into biogas and the residual substrate can be separated into a liquid, high value organic fertilizer and a solid fraction suitable for high value chemicals production. Thus, the term waste product is more or less non-existing in the context of LCF biorefinery (Thomsen, 2005).

LCF biorefinery is recognized as the future biorefinery system because its raw materials are huge and available at low costs, contrary to other existing biorefinery system where a material and product diversity is non-existence. So they are highly sensitive to rising prices for raw materials such as corn or grain (Lyko et al., 2009). This is apparent as biorefinery of the future needs a large biomass infrastructural piece in front which will enable a continuous production process of its products to meet the market demands. Such a system has been implemented in commercial and demonstration phase around the world (Bacovsky et al., 2010).

Raw material for LCF biorefinery

Lignocellulosic plant biomass

Lignocellulose is made up of three-dimensional polymeric composite formed by plant biomass as structural complex material. It composed primarily of cellulose, hemicelluloses, and lignin and smaller fractions of proteins, oil, wax, and ash. Cellulose fraction is often the most abundant part of lignocellulose plant biomass with hemicellulose and lignin in diverse

proportions depending on the plant/crop type. Those three main constituents of lignocellulose are closely interconnected to form the structural framework of plant cell wall (Jørgensen et al., 2007). The structural architecture and chemical composition of lignocellulose play a significant role in its resistance to decomposition and degradation by microorganisms (Kuhad et al., 1997). Thus, it is important to clearly understand the nature, structure and composition of the polymeric composite of lignocellulose plant biomass in order to efficiently convert them into valuable biobased products. Chemical compositional contents of some lignocellulosic biomass are depicted in Table 1.

Cellulose

Cellulose is the primary structural polysaccharide of plant cell walls, and often being the most abundant biological molecule on earth. Cellulose fraction of lignocellulose plant biomass is a homogenous polymer of D-glucose units linked together by β-1, 4-glucosidic bonds. The degree of polymerization (DP) of native cellulose is in the range of 7.000-15.000 (Berg et al., 2002; Ward and Moo-Young, 1989).

$$DP = \frac{\text{Molecular weight of cellulose}}{\text{Molecular weight of one glucose unit}}$$

Cellulose molecules are totally linear and have a strong tendency to form intra and intermolecular hydrogen bonds. Bundles of cellulose molecules are thus aggregated together in the form of microfibrils, in which highly ordered crystalline domains alternate with less ordered amorphous regions (Sjöström, 1981). The cellulose molecules form extremely ordered crystalline regions through parallel orientation, but are well-connected with amorphous regions of more disordered structure (Lynd et al., 2002). The cellulose structure laterally with the intermolecular hydrogen bonds gives cellulose high tensile strength, makes it insoluble in most solvents and is partly responsible for the resistance of native cellulose against microbial attack (Jørgensen et al., 2007). The act of resistant to depolymerization makes it rather difficult for hydrolyzing enzymes to access its fractions. Therefore, release of glucose from cellulose faces significant technical challenges.

Hemicellulose

Hemicelluloses, the second most abundant biopolymers on earth and its conversion to ethanol could provide an alternative liquid fuel source for the future (Jeffries, 2006). It involves a group of biopolymers that are closely linked with cellulose and are usually a mixture of heterogeneous polysaccharides that have a complex composition and structure (Stepan et al., 2012). The content of hemicelluloses varies widely, depending on plant genus, cell type, growth conditions, method of extraction and storage. Like plant belonging to grass family (Poales), have the branched polymer of glucuronoarabinoxylan (GAX) as the most common hemicelluloses (Carpita, 1996). Hemicelluloses were originally believed to be intermediates in the biosynthesis of cellulose. The main components of its heteropolysaccharides are pentoses (xylose, arabinose) as the dominant sugars, hexoses (mannose, glucose, galactose) and small amounts of organic acids such as uronic acid, which occurs in following forms: glucuronic acid (and its 4-*O*-methyl ether), and galacturonic acid (Saha and Bothast, 1997). Xylose is the predominant pentose sugars available from the hemicellulose of most hardwood materials, whereas arabinose can constitute a significant amount of the pentose sugars available from various agricultural residues and other herbaceous crops (Balat et al., 2008).

Hemicelluloses usually have an average of degree of polymerization 100 to 200 depending on their type and origin (Kuhad et al., 1997). In contrast to cellulose, which is crystalline and strong , hemicelluloses have a random, amorphous, branched structure with little resistance to depolymerization, and are relatively easy to hydrolyze to their sugar monomers by dilute acids (Hamelinck et al., 2005).

Lignin

Lignin is a complex polymer of phenylpropane units and methoxy groups, linked in a tri-dimensional structure which is particularly difficult to biodegrade. It constitutes the most abundant non-carbohydrate fraction in lignocellulose. It is made-up of three aromatic monomers, which are *trans-p*-coumaryl alcohol, *trans-p*-coniferyl alcohol and *trans-p*-sinapyl alcohol and are joined via alkyl-aryl, alkyl-alkyl and aryl-aryl ether bonds (Zaldivar

et al., 2001). Lignin is most recalcitrance component of plant cell wall and protects the plant from physical and microbial degradation (Hendriks and Zeeman, 2009).

Table 1. Cellulose, hemicellulose and lignin content in most common lignocellulosic materials.

	(% of total dry weight)		
Lignocellulosic materials	Cellulose	Hemicellulose	Lignin
Hardwoods stems	40-50	24-40	18-25
Softwood stems	45-50	25-35	25-35
Wheat straw	33-40	20-25	15-20
Grasses	25-40	35-50	10-30
Corn cobs	45	35	15
Nut shells	25-30	25-30	30-40
Paper	85-99	0	15
Switch grass	30-50	10-40	5-20
Sorted refuse	60	20	20
Leaves	15-20	80-85	0
Waste papers from chemical pulps	60-70	10-20	5-10
Cotton seed hairs	80-95	5-20	0
Primary wastewater solids	8-15	NA[a]	NA[a]
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Sugarcane bagasse	25-45	28-32	15-25
Rice straw	29.2-34.7	23-25.9	17-19
Corn stover	35.1-39.5	20.7-24.6	11.0-19.1
Bamboo	49-50	18-20	23

Sources: Kumar et al. (2009); McKendry, (2002); Menon and Rao, (2012); Sun and Cheng, (2002). [a]NA – not available.

The lignin content of biomass largely depends on the plant type, for example, grasses in general have the lowest lignin content in comparison to softwoods where the lignin content is the highest (Jørgensen et al., 2007). Therefore, the higher proportion of lignin in the lignocellulosic biomass, the higher is its resistance to chemical and enzymatic degradation. It restricts hydrolysis by shielding cellulose surfaces and inactivating enzymes (Taherzadeh and Karimi, 2008). Lignin is insoluble in water and most often resistant in acidic conditions, but can be altered under alkali condition.

Conversion process routes for LCF biorefinery

Background of biomass pretreatment methods

The characteristic of native lignocellulosic biomass such as its crystallinity, presence of lignin and hemicellulose, inaccessible surface area, degree of cellulose polymerization, and degree of acetylation of hemicelluloses makes it resistant to enzymatic degradation. Therefore, to economically convert carbohydrates in lignocellulosic biomass into fermentable sugars, a pretreatment step is necessary to render the cellulose fraction accessible to hydrolyzing enzymes (Wyman, 1996). The goal of pretreatment is to decrease the crystallinity of cellulose, increase biomass surface area, remove hemicellulose, and break the lignin seal (Taherzadeh and Niklasson, 2004) as illustrated in Figure 4. This process changes the biomass structure and improves downstream processing.

Various pretreatment methods have been proposed to make lignocellulosic biomass susceptible to enzymatic and microbial conversion (Galbe and Zacchi, 2002; Hendriks and Zeeman, 2009), such as wet oxidation (Schmidt and Thomsen, 1998), dilute acid hydrolysis (Saha et al., 2005a), and steam explosion (Ballesteros et al., 2006), and many more. However, for comprehensive review of all the available pretreatment methods, the reader is kindly advised to see other review articles (Mosier et al., 2005; Taherzadeh and Karimi, 2008; Zheng et al., 2009; Kumar et al., 2009). Pretreatment is among the most costly steps in biochemical conversion of lignocellulosic biomass, accounting for up to 40% of the total processing cost (Eggeman and Elander, 2005; Wyman et al., 2005b; Lynd, 1996; Zhang et al., 2009). Thus, cost-effective pretreatment of lignocellulosic biomass is a major challenge

of cellulosic biofuels and bioproducts technology research and development in recent years (Hamelinck et al., 2005).

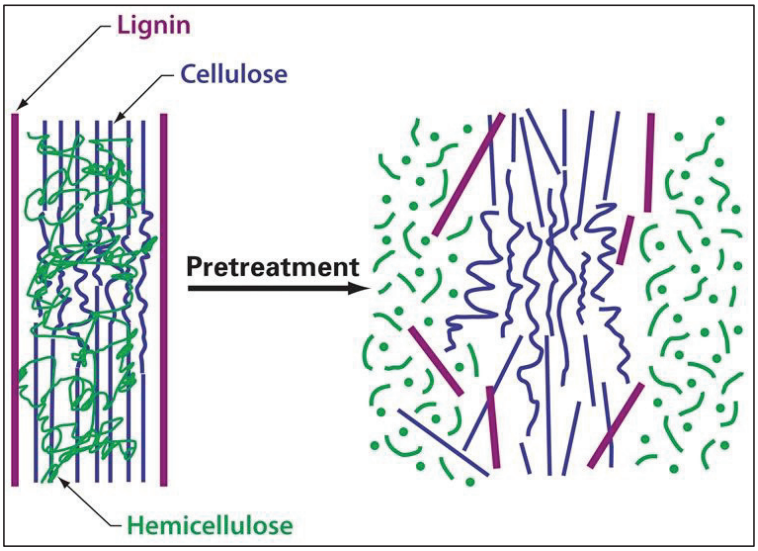

Fig. 4. Schematic overview of the matrix of lignocellulosic polymers in which the pretreatment separated hemicellulose and lignin from cellulose enabling effective enzymatic digestibility (Mosier et al., 2005).

The choice of a suitable pretreatment method and the adjustment of the pretreatment parameters are crucial for the efficiency of the subsequent conversion of any biomass in a biorefinery concept. Hence, an effective pretreatment should meet the following conditions: (1) maximize the enzymatic convertibility of cellulose fractions (2) avoids the need for reducing the size of biomass particles (3) avoid the formation of fermentative inhibitor (4) minimizes energy demands and limits cost (5) avoid destruction of hemicellulose and cellulose fractions of biomass (6) minimizes waste generation (7) should facilitate recovery of lignin and other constituents for conversion to valuable co-products and simplify downstream processing (8) utilize little or no chemical and using low cost chemicals (9) minimizes the need for detoxification of subsequent enzymatic hydrolysis and fermentation

and above all, pretreatment results must be weighed against their impact on the ease of operation and cost of the downstream processing steps and the trade-off between several costs, including operating costs, capital costs, and biomass costs (Mosier et al., 2005; Zheng et al., 2009). Till date, no pretreatment method has been identified as the most efficient because their mode of action often varies, but they all pursue one goal; to produce higher fermentable sugars irrespective of biomass type. Therefore, the above-mentioned conditions should be comprehensively considered as a basis when comparing various pretreatment methods. On the other hand, pretreatment process parameters should be tailored to the specific biomass compositional structures and with a view to all the potential sugars which can be produced.

Wet oxidation pretreatment

Wet oxidation pretreatment method has been applied in laboratory and pilot-scale process for fractionation of several lignocellulosic biomass such as wheat straw, sugarcane bagasse, softwood, corn stover, clover-ryegrass mixtures and rice husk (Ahring and Westermann, 2007; Westermann and Ahring, 2005; Varga et al., 2003; Martin et al., 2008; Banerjee et al., 2009; Palonen et al., 2004). Wet oxidation make use of oxidizing agent such as H_2O_2, water and air or oxygen at pretreatment temperatures around 180-200^{o}C for 5-30 min (McGinnis, 1983). The process is normally carried out at 5-20% dry matter. Wet oxidation is an exothermic process, and thus, it becomes self-supporting with respect to heat while the reaction is initiated (Taherzadeh and Karimi, 2008, Schmidt and Thomsen, 1998). It has been documented that the most crucial parameters in wet oxidation process is the temperature followed by residence time and oxygen pressure (Schmidt and Thomsen, 1998). Wet oxidation is an effective pretreatment method in breaking the lignin seal and separating hemicellulose from cellulose thereby facilitate the solubilization of hemicellulose fractions while the lignin is mainly oxidized and cellulose is made susceptible to hydrolyzing enzymes. However, under this process, some of the lignin together with hemicelluloses is partially oxidized to low molecular weight carboxylic acids, CO_2, and water (Klinke et al., 2002), as the main reactions are the formation of acids from hydrolytic processes, as well as oxidative reactions (Taherzadeh and Karimi, 2008). The

combination of alkaline and wet oxidation has been proven to prevent the formation of degradation products such as furfural and hydroxymethylfurfural (HMF) as reported by Bjerre et al. (1996). Wet explosion similar to wet oxidation is a novel pretreatment method recently developed to fractionate lignocellulosic biomass at high dry matter concentration (Rana et al., 2012; Sørensen et al., 2008; Georgieva et al., 2008). This method uses a combination of physical and chemical pretreatment methods and the operating condition is around 170^{o}C and 20 bars. It is also flexible in additives (H_2O_2, oxygen and air) and biomass input such as wheat straw and sugarcane bagasse. The main principles are the injection of oxidizing agents at a desired temperature while the residence time is initiated and the termination of the pretreatment by flashing the biomass into a flash tank, called "explosion" which usually results in a sudden drop in temperature and pressure. Finally, wet oxidation and wet explosion can easily be carried out as a continuous process providing the path to scale-up at commercial level.

Dilute acid pretreatment

Dilute acid pretreatment is widely recognized in the field of biofuels production for pretreatment of lignocellulosic biomass. High yields of sugars from lignocellulosic biomass can be achieved from dilute acid pretreatment. The mode of action is hemicellulose removal and thus, enhances the digestibility of cellulose in the residual solids. The process is mainly carried out under high temperature and low acid concentration and the most widely used acid under this process is sulfuric acid (Kim et al., 2011; Saha and Bothast, 1999; Foston and Ragauskas, 2010). However, other acids such as hydrochloric acid, phosphoric acid and nitric acid have been extensively applied (Herrerat, et al., 2003; Vazquez et al., 2007; Taherzadeh and Karimi, 2007a). High pretreatment temperature with dilute sulfuric acid can achieve high reaction rates and significantly improve cellulose hydrolysis (Esteghlalian et al., 1997), but moderate temperature is more desirable to release hemicellulose sugars, because hemicellulose is amorphous. The acid catalyzes the breakdown of hemicellulose long chains to form shorter chain oligomers and then to sugar monomers and it can further degrade the monomeric sugars to furfurals and other degradation products (Wyman et al., 2005a). Normally, the acid is mixed or contacted with

biomass and held at temperatures of 160-220°C for periods ranging from minutes to seconds (Mosier et al., 2005). However, this process has its own drawback, such as the corrosion that mandates expensive materials of construction, formation of degradation products, and acidic prehydrolyzates that must be neutralized before the sugars proceed to fermentation (Zheng et al., 2009; Sun and Cheng, 2002; Kumar et al., 2009).

Taherzadeh and Karimi, (2008) reported that dilute acid pretreatment is not effective in dissolving lignin, but it can disrupt lignin and enhance digestibility of cellulose and that around 100% hemicellulose removal is possible under this method. Furthermore, Lavarack et al. (2002) investigated sugarcane bagasse subjected to dilute sulfuric acid or hydrochloric acid pretreatment at temperatures around 80-200^{o}C for 10-2000 min. It observed that hydrochloric acid was less active for the degradation of xylose compared to sulfuric acid and that almost 80% of theoretical xylose was achieved from bagasse. In a similar manner, Saha et al. (2005b) found 60% yield of total sugars based on total carbohydrate content of rice hulls pretreated with 1.0%, v/v dilute sulfuric acid and subsequent enzymatic hydrolysis. They further mentioned that no furfural and HMF were produced, which are normally considered as inhibitory compounds to fermentative microbes.

Steam explosion pretreatment

Steam explosion is the most commonly used method for pretreatment of lignocellulosic biomass because it does not require the addition of chemicals (uncatalyzed steam explosion). The mode of action involves treatment of chipped biomass with high-pressure saturated steam, followed by a sudden drop in pressure, which makes the materials undergo an explosive decompression. This sudden pressure release reduces the temperature and quenches the reaction at the end of the pretreatment. Steam explosion is typically initiated at temperatures around 160-260^{o}C (corresponding pressure, 0.69-4.83 MPa) for several seconds to a few minutes before the material is exposed to atmospheric pressure (Boussaid et al., 1999; Kurabi et al., 2005; Varge et al., 2004; Ruiz et al., 2006; Carrasco et al., 1994; Josefsson et al., 2002; Ballesteros et al., 2004; Mes-Hartree and Saddler, 1983; Sun et al., 2004; Laser et al., 2002; Cullis et al., 2004).

The conversion rate of carbohydrates can be effectively increased during steam explosion (Varge et al., 2004), thereby enhances the potential of cellulose hydrolysis and extensively hydrolyzed hemicellulose to fermentable sugars (Mosier et al., 2005; Fernandez-Bolaños et al., 2001). The moisture in the biomass hydrolyzes the acetyl groups of the hemicellulose fractions, forming organic acids such as acetic and others. The acids, in turn catalyze the depolymerization of hemicellulose, releasing xylose and small amounts of glucose. Under severe conditions, the amorphous regions of cellulose may be hydrolyzed to some degrees. Applying high temperatures and pressures, however, can enable the degradation of xylose to furfural and glucose to HMF, and transformation of lignin. These degradation products are considered inhibitory to microbial growth during ethanol fermentation (Menon and Rao, 2012; Mok and Antal Jr., 1992; Mackie et al., 1985; Bobleter et al., 1981). Cantarella et al., (2004a) reported that steam explosion pretreatment of poplar wood at 214^{o}C for 6 min was efficient to achieve approximately 91% theoretical yield of ethanol, although, some lag phases were observed as a result of the associated inhibitory compounds produced during the pretreatment. Generally, steam explosion is a unique pretreatment method because it requires low energy input and can operate with or without chemicals.

Separation process

Separation of pretreated biomass into solid and liquid fractions is necessary in order to obtain high valuable fractions of the biomass slurry depending on the process configuration and the end products choice of interest. Different kinds of separation methods and technologies exist such as filtration and membrane separation (Huang et al., 2008; Vane, 2005; Peng et al., 2012). Biomass separation after pretreatment enables products removal and purifications. The resulting biomass slurry contains all the fractions of lignocellulosic biomass (cellulose, hemicellulose, and lignin) both in form of polymeric and monomeric including different types of degradation products. These materials are therefore removed from each other to some degree by separation method.

Usually, about 80-90% of dissolved organic matter is transferred to the liquid, which mainly contains the hemicellulose sugars and small fractions of glucose, lignin products,

and furans. On other hand, the solid fraction normally contains at least 25% dry matter, where the most part of cellulose and lignin fraction is deposited depending on the pretreatment method applied. Obtaining high valuable materials via separation can go a long way to reduce the process costs in biorefinery systems. It also enables process flexibility and diversifications, meaning that such a system can integrate different production processes and be able to utilize all the fractions of pretreated biomass to produce a wide range of biobased products in order to meet the market demand and supply.

Formation of by-products during biomass pretreatment

Depending on process severity, carboxylic acids, furan derivatives (furfural and 5-hydroxymethyl furfural-HMF), and phenolic compounds were generated during pretreatment of lignocellulosic biomass. These products are considered potential fermentation inhibitors (Saha, 2004; Klinke et al., 2004). The most investigated inhibitors towards microbial growth includes furfural, HMF, acetic acid, formic acid, levulinic acid, vanillic acid, and phenol (Bellido et al., 2011; Martin et al., 2006; Luo et al., 2002; Xiros et al., 2010; Weil et al., 2002). Those inhibitory compounds significantly affect the overall cell physiology and when in high concentrations, it can result in decreased viability, ethanol yields, and productivity. At more severe pretreatment conditions, xylose is degraded to furfural while HMF is formed from hexose degradation, and phenolic compounds are generated from partial breakdown of lignin (fig. 5). Furfural is further degraded to formic acid likewise HMF to levulinic (Palmqvist and Hahn-Hägerdal, 2000).

The formation of these compounds during pretreatment of biomass has been reported elsewhere in literatures to be associated with high pretreatment temperature and acid concentrations (McGinnis et al., 1983; Rivard et al., 1996; Jacobsen and Wyman, 2000; Agbor et al., 2011; Mosier et al., 2005). Dehydration of hexose and pentose sugars under thermal and acidic conditions enhances the formation of furfural and HMF in the liquid fraction of the pretreated biomass (Martin et al., 2007). It has been reported that these compounds not only reduces the sugar yield, but pose a serious threat to fermentative microorganism (Pedersen and Meyer, 2010; Buchert et al., 1990; Clark and Mackie, 1984). The inhibitory effect of furans (Palmqvist et al., 1999a; Taherzadeh et al., 2000; Palmqvist

et al., 1999b), aliphatic acids (Taherzadeh et al., 1997) and aromatic compounds (Larsson et al., 2000; Ando et al., 1986) during fermentation with *Saccharomyces cerevisiae* has been investigated with respect to sugar consumptions.

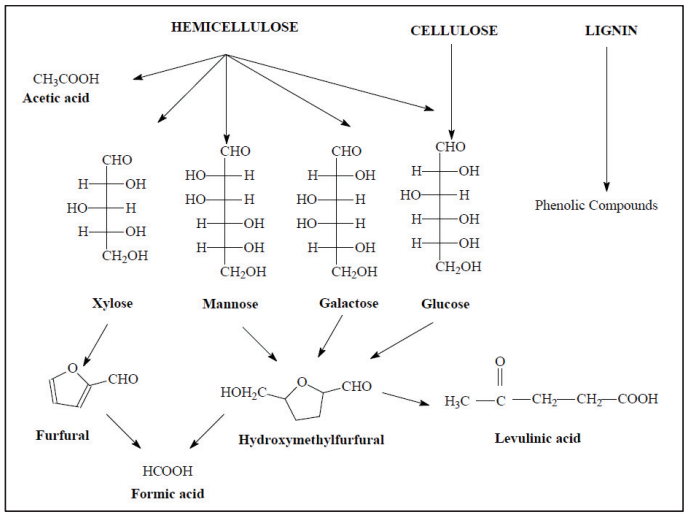

Fig. 5. Reactions occurring during hydrolysis of lignocellulosic materials (Palmqvist and Hahn-Hägerdal, 2000).

However, these inhibitory compounds can be detoxified in order to adapt the microorganisms to utilize the available sugars to ethanol. Overliming and neutralization are some of the proposed methods to carryout hydrolysate detoxification (Cantarella et al., 2004b; Chandel et al., 2007a). But, performing hydrolysate detoxification is often energy demanding and can elevate the process cost of the ethanol production process. In order to make lignocellulosic ethanol production more economically feasible, the hydrolysates arising from the pretreated lignocellulosic biomass should be able to ferment to ethanol without the need for further detoxification.

Enzymatic hydrolysis

Enzymatic convertibility of cellulose is one of the most important factors to evaluate the efficiency of a specific pretreatment method for production of biofuels, mostly ethanol (Varge et al., 2003) as it reveals the efficiency of enzymatic hydrolysis specifically for a certain enzyme mixture on the specific hydrolysate. It is the second step after pretreatment for a cellulosic bioconversion processes and it hydrolyzed the polymers of cellulose and hemicellulose fractions into fermentable sugars, such as glucose, xylose, arabinose and etc. as its end-products (Taherzadeh and Niklasson, 2004). There are several commercial cellulases producers around the world, such as Novozymes, Genencor (DuPont) and Codexis, and most of them are produced from *Trichoderma spp*., with small produced by *Aspergillus niger*. The major factors affecting enzymatic hydrolysis of cellulose includes among others pretreatment method, substrate concentration, hydrolysis condition (temperature, pH, and mass transfer), and enzyme activity. However, several studies have shown that the optimum temperatures and pH for cellulases are usually in the range of 40-50^{o}C and pH 4-5 (Taherzadeh and Karimi, 2007b).

The lignin content of biomass is also one of the most important factors hindering hydrolysis of biomass by cellulases and hemicellulases (Van Dyk and Pletschke, 2012). It is known that the enzymatic convertibility of cellulose into glucose monomers is mostly accomplished by synergistic action of three groups of cellulases: exo-1,4-β-D-glucanases, EC 3.2.1.91 and EC 3.2.1.176 (cellobiohydrolase), which move processively along the cellulose chain and cleave off cellobiose units from the reducing ends, endo-1,4-β-D-glucanases, EC 3.2.1.4, which hydrolyze internal β-1,4-glucosidic bonds randomly in the cellulose chain and β-glucosidases, EC 3.2.1.21, which hydrolyze cellobiose to produce glucose. In addition, β-glucosidases not only produce glucose from cellobiose but also reduce cellobiose inhibition, thereby permitting the cellulolytic enzymes to perform more efficiently. Therefore, it is required that the enzyme mixtures must include all the three classes of cellulolytic enzymes in order to obtain a complete hydrolysis and better yield of end products (Demain et al., 2005; Jørgensen et al., 2007; Bayer et al., 1998; Wilson, 2009; Schwarz, 2001; Wilson, 2011).

Regardless of enzyme mixtures, the dosage in hydrolysis media should be at a minimal range to achieve the economic viability of the process. Although, hemicellulose contain reasonable amount of fermentable sugars, its enzymatic convertibility is more complex to that of cellulose. It is mainly composed of a mixture of five and six sugar units which requires several different enzymes to hydrolyze it. The hemicellulytic enzymes that can degrade hemicellulose includes but not limited to endo-1,4-β-D-xylanases, EC 3.2.1.8, which cleave the xylan backbone into shorter oligosaccharides, β-D-xylosidase, EC 3.2.1.37, which cleave xylooligosaccharides from the non-reducing end and produce xylose, endo-β-D-mannanase, which attack internal bonds in mannan and β-D-mannosidase, which clave mannooligosaccharides to mannose and many ancillary enzymes such as α-L-arabinofuranosidases, α-glucuronidase, α-galactosidase, feruroyl esterase, acetyl xylan esterase, which removes the side groups in the substrate (Jørgensen et al., 2003; Beg et al., 2001; Shallom et al., 2003; Meyer et al., 2009). Therefore, it is crucial to modify the pretreatment process parameters to the specific biomass compositional structure to produce fermentable sugars which will evict subsequent enzymatic hydrolysis of hemicellulose fractions and at the same, reduce the costs of the process.

Ethanol fermentations

The fermentation of lignocellulosic materials is carried out after pretreatment and subsequent enzymatic hydrolysis of either the separated solid fractions or liquid fractions and can also be the whole hydrolysate to fermentable sugars. This hydrolysate normally contains various hexoses, mainly glucose, and pentoses, mainly xylose, and various inhibitory compounds depending on the substrate and the applied pretreatment method. Both hexose and pentose sugars are fermented to ethanol and other valuable biobased products under anaerobic/aerobic conditions. At present, the ethanol fermentation is mostly performed with the yeast *Saccharomyces cerevisiae* because of its well-known characteristics, high tolerant, robustness and high ethanol yield. However, this yeast have ability to metabolize only hexose sugars, while the pentose sugars can be fermented by other organisms such as *Pichia stipitis* and *Candida shehatae* (Parekh and Wayman, 1986; Taniguchi et al., 1997; Moniruzzaman, 1995; Zhu et al., 2009). On the other hand, *S.*

cerevisiae can be genetically engineered to ferment pentoses (Kim, et al., 2005; Schneider et al., 1981; Slininger et al., 1982; Jeffries et al., 1982; Van Zyl et al., 2007). Currently, there are several process routes to ferment pretreated lignocellulosic materials to ethanol by employing different kinds of ethanologenic strains. This includes among others separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharifictaion and co-fermentation (SSCF), and consolidated bioprocessing (CBP) see Figure 6 (Lynd, 1996; Lynd et al., 2002).

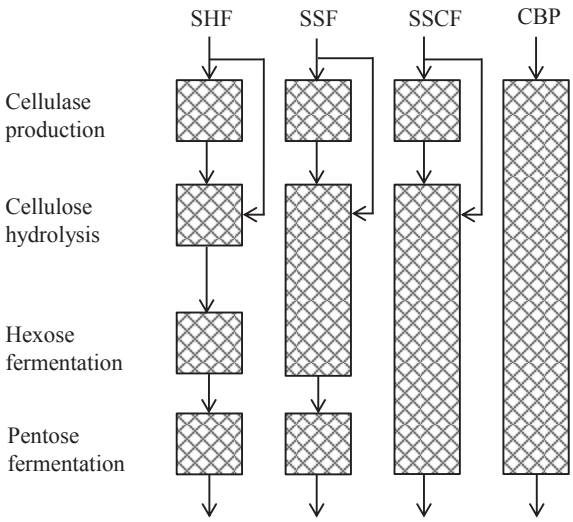

Fig. 6. Different processing routes that can be employed during biomass conversion to valuable products (Lynd, 1996; Lynd et al., 2002).

Separate hydrolysis and fermentation: SHF involves four separate process steps that make use of separate bioreactors. Under this configuration, cellulose or hemicellulose is first hydrolyzed enzymatically into sugar monomers, and subsequently fermented to ethanol and chemicals. The advantage of this process is that each step can be individually optimized to reach their optimal conditions and improve its process performance. The major disadvantage of this process is that the generated cellobiose can significantly inhibit the

performance of cellulase (Reese, 1963), and β-glucosidase can be also inhibited by glucose (Holtzapple et al., 1990), as a result, it requires the use of lower solids concentrations at higher enzyme loadings to achieve high ethanol yields. Low solids concentrations, however, will result in low ethanol yield, thus, increasing the cost of downstream process (Wyman, 1996).

Simultaneous saccharification and fermentation: SSF consolidates hydrolysis and fermentation of hexose sugars in a single step, which means that the sugars that is released is simultaneously consumed by the fermenting organisms and inhibition of cellulytic enzymes is therefore prevented. This configuration reduces the number of steps in the process, and is thus, a promising route for conversion of cellulosic materials to ethanol and chemicals (Lynd et al., 2005; Wyman, 1994; Erdei et al., 2010), since all the process steps is integrated in one reactor, and therefore, making the process of SSF more cost effective in comparison to SHF process (Olofsson et al., 2008). The major drawback of this configuration is the optimal working condition (temperature). The optimal temperatures for cellulases and the fermenting organisms are not the same, therefore, the chosen temperature is a compromise, normally the temperature is selected to fervor the fermenting organisms, since a liquefication step is usually done during hydrolysis for some hours in order to ensure a proper substrates mixing (Eklund and Zacchi, 1995; Demain et al., 2005).

Simultaneous saccharifictaion and co-fermentation: A configuration which is seen as more SSCF where the hydrolysis and the fermentation of hexoses and pentoses take place in one process step. This configuration is normally performed by using microorganisms capable of utilizing both hexoses and pentoses to ethanol and chemicals. The ethanologenic strains such as *S. cerevisiae* and *P. stipitis* are by far the most used, where the *yeast P. stipitis* is first added to the substrates and the *S. cerevisiae* is used to complete the fermentation in one reactor (sequential fermentation). It can also applied genetically engineered organisms capable of utilizing both hexoses and pentoses to ethanol (Sanchez and Cardona, 2008; Cardona and Sanchez, 2007). This process is more advance to SSF and SHF process with respect to better ethanol yields, cost effectiveness, and high rates conversion (Joshi et al., 2011; Chandel et al., 2007b; De Bari et al., 2004; Grootjen et al., 1991).

Consolidated bioprocessing: The concept of consolidated bioprocessing CBP is aimed at consolidating cellulase production, hydrolysis and fermentation in one process step, which means that all the four process steps as seen in SHF is integrated in a single step. Lynd, (1996) pointed out that no capital or operation expenditures are required for enzyme production within this concept. The objective is to create a single microorganism that is capable of performing these steps simultaneously (Lynd et al., 2005; Van Zyl et al., 2007; Cardona and Sanchez, 2007). The development of bioconversion technologies has recently been shifted towards CBP because it is believed that CBP has the largest potential of reducing process materials, production costs and higher conversion efficiencies than SSF or SSCF based processes. There are two strategies to create CBP microorganisms; (1) naturally occurring cellulytic microorganisms can be genetically engineered to enhance product-related properties such as high ethanol yield and titer, and (2) a non-cellulytic microorganism that gives high yields can be altered by genetic engineering to express heterologous cellulase system enabling cellulose utilization (Lynd et al., 2005). CBP offers the opportunity of producing low cost bioproducts, but the right microorganisms that possess all the needed features in CBP configuration are not readily available, on the other hand, a number of bacteria and fungi that exhibits some of the needed features have been identified (Van Zyl et al., 2007; Lynd et al., 2002). Wyman, (1994) reported that most of the studies shows that bacterium *Clostridium themocellum* is used for enzyme production, cellulose hydrolysis, and its conversion to ethanol, however, the co-fermentation applying *Clostridium thermosaccharolyticum* permits the simultaneous conversion of monomeric hemicellulose sugars to ethanol after hydrolysis. This shows that the right candidate (microorganism) that exhibit the whole combination of features required for the development of a CBP is still under investigation (Cardona Sanchez, 2007).

Anaerobic digestion

In the context of biorefinery concept, implementing an integrated production process will to large extent reduce the process costs and make it more economically feasible. This will enable the process of biorefinery to be more flexible, by this reduce market and technical risk that might arise when focuses in one particular product. Co-products,

however, will in long-term support biorefinery economics when the principle of diversity is maintained. With regards to ethanol production, the effluent from the process normally contains high-value organic fractions, which can be further converted to valuable biobased products and energy. They are many efficient ways to convert these effluent materials into bioproducts, one way that has gain much attention over the years is by anaerobic digestion (AD) to produce methane biogas and organic fertilizer. The integration of bioethanol and biogas production in one process plant has been demonstrated both in pilot and lab-scale around Europe (Mohammad Karimi, 2008; Lissens et al., 2004; Uellendahl and Ahring, 2010; Oleskowicz-Popiel et al., 2012; Jeihanipour et al., 2010). Anaerobic digestion (AD) process is said to be a complex microbiological process, during which organic substrates are decomposed into biogas and microbial biomass in the absence of oxygen (biogas, a mixture of carbon dioxide and methane, a renewable energy source) (Chen et al., 2008). It's a complex process that involves interaction between many consortia of microorganisms and each consortium thrives optimally at a given set of chemical and physical conditions. AD occur in a divers environments, such as marine and fresh water sediments, and in the intestinal tract of ruminants or sewage sludge. The degradation processes can be categorized into four major steps; hydrolysis, acidogenesis, acetogenesis, and methanogenesis as represented in Figure 7.

Hydrolysis is the first step in AD process, during which complex organic matter is broken down into shorter chain mono and oligomers by exoenzymes (cellulases, lipases and proteases) excreted by fermentative bacteria into various compounds, which can be transported through the cell membrane. However, degrading complex lignocellulosic materials has been recognized as the rate-limiting step during hydrolysis, since exoenzymes are not able to degrade native lignocellulosic biomass into its monomers (Boe, 2006).

A process where acidic bacteria degraded the decomposed compounds into fermentation products (volatile fatty acids (VFA), ethanol, hydrogen, lactic acid and carbon dioxide) is termed acidogenesis. In acetogenesis step, the fermentation products are oxidized to acetate, carbon dioxide and hydrogen, which are with no doubt the substrates for methanogenic bacteria. Methanogenesis is very crucial during the AD process, since this is where the end products are derived. Methane and carbon dioxide production from

intermediate products is accomplished by methanogenic bacteria. Almost 30% of the methane is generated from the conversion of hydrogen and carbon dioxide (hydrogenotrophic process), and around 70% of the methane is produced from the conversion of acetate (acetoclastic) (Boe, 2006).

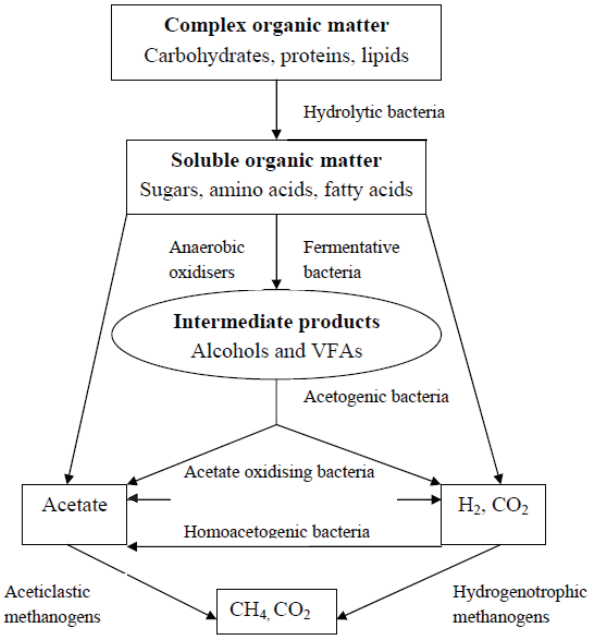

Fig. 7. Carbon flow diagram of the biogas production processes (Angelidaki et al., 2002).

Anaerobic digestion conditions are important to achieve high biogas yields and to run the process accurately and efficiently. Therefore, a number of process parameters that affect AD process should be monitored and maintained in the optimal range to gain economically feasible process. Some of the crucial process parameters are: pH, temperature, volatile fatty acids (VFA), macro and micro nutrients, and etc.

The pH is considered as one of the crucial parameter for microbial growth. The methanogenic bacteria exert the highest intolerance towards fluctuations in the pH and the optimum pH for hydrolytic and acidogenic bacteria is normally at 6, but to that of

methanogenic bacteria, their optimum pH is usually in the range of 7-8 (Chen and Hasimoto, 1996), hence, the process is inhibited if the pH is below 6 and above 8.3. Further, pH can also affect the dissociation of other compounds such as ammonia, sulfide and organic acids. Ammonia produced during protein degradation results in an increase of the pH while VFA and carbon dioxide production during the acidogenesis phase might reduce the pH (Angelidaki and Ahring, 1993). However, monitoring pH alone can give a false impression of the process condition, as the pH is mainly controlled by the bicarbonate buffer system in AD process. Thus, pH should not be used as a stand-alone monitoring parameter.

Anaerobic digestion can be performed in a wide range of temperatures from psychrophilic, mesophilic, and thermophilic. However, because of the strong reliance of temperature on the digestion rate, temperature is perhaps the most essential parameter to maintain in a desired range. Anaerobic microbes can survive in a wide range of temperatures, from (12 ºC to18 ºC) the psychrophilic range, (25 ºC to 40 ºC) the mesophilic range, and (55 ºC to 65 ºC) the thermophilic range. The optimum temperature for mesophilic digestion is 35ºC and a digester must be maintained between 30ºC and 35ºC for most favorable functioning. Thermophilic anaerobic digestion thrives most at temperature of 60 ^{o}C and is normally considered as more efficient process with respect to organic matter removal and gas production. It is highly recognized as a process where the pathogen content is extensively reduced to that of mesophilic process. The rate of anaerobic digestion process is measured by gas production rates, growth rates, and substrate degradation performance. Thermophilic process allows higher loading rates and achieves a higher rate of pathogen destruction as well as higher degradation of the substrate. This growth rate is mainly dependent on temperature and the rate increases with increasing temperature (Van Lier, 1995), which is why thermophilic condition is more desirable. Though, most of the biogas producing plants operates at mesophilic conditions, because the process is more vigorous and can tolerate greater changes in the environmental conditions including temperature.

Volatile fatty acids (VFA) concentrations have been recommended as a parameter to monitor AD process because they are the main intermediate products. It also provides a fast

indication for upcoming process instability as the elevated VFA concentration can indicate instability of the process. Normally, under acidogenesis around 30% of its compounds are volatile fatty acids (fatty acids with a carbon chain of six carbons or fewer, e.g. acetate, propionate, butyrate). These intermediate products may have inhibitory effect on the process, if they found in higher concentrations. Acetate is the main intermediate and its accumulation reduces the metabolic activity of butyrate and propionate degrading bacteria. However, every biogas plant operates differently, the concentration of VFA maybe optimal for one plant and it may not be the case for the other. Therefore, VFA concentrations should be integrated with other process parameters in order to gain a full control of AD process (Pind et al., 2003).

The maintenance of optimal microbiological activity in the digester is crucial to gas production, consequently is related to nutrient availability. Therefore, some nutrients should be present and available in the medium for bacterial growth. Oxygen, nitrogen, carbon, and hydrogen are main constituents in organic material. Ammonia and sulfide are the sulfur and nitrogen sources for the microorganisms in the anaerobic reactor. Phosphorus is mainly seen in nucleic acids, phospholipids, ATP, GTP, NAD, AND FAD, while sulfur is necessary for synthesis of amino acids, cysteine and methionine. Calcium, magnesium, potassium, and iron are required as fraction of metal complexes and also as cofactors for enzymes activity. The C/N ratio, i.e. the connection between carbon and nitrogen has to be in equilibrium to secure a stable process. The C/N ratio can be judiciously manipulated by combining materials low in carbon with those that are high in nitrogen, and vice versa (Angelidaki et al., 2002; Al Seadi, 2001).

LCF biorefinery products, application and utilization

Bioethanol

Biorefinery products are mainly classified into two main groups: energy products and material products. The choice of its product portfolio should depend on the market demands. Therefore, the biorefinery system should be able to integrate different process line that can produce high-value products on a continuous process basis to meet the market

demands. There should be at least one large-scale main products, for example fuel or bulk chemical, for cost recovery and several high value coupled products, like fine chemicals, for diversity and returns. Changes that bring improved flexibility and diversification by capturing the benefits of economies of scale can add new value to such a system and help establish a competitive advantage over fossil products. We therefore, highlight some of such biobased products and their potential applications.

Ethanol or ethyl alcohol, has been identified as one of the most interesting synthetic oxygen-containing organic chemical because of its unique combination of properties as a solvent, a beverage, an antifreeze, and more especially due to its versatility as a chemical intermediate for other chemicals. Ethanol is an industrial chemical which has high significant utilization. It can be used in the transportation sectors as well as in production of pharmaceutical products, dyestuffs, perfumes and numerous products. Ethanol under ordinary condition is a volatile, flammable, clear, colorless chemical compound. The largest bioethanol producers in the world are the USA and Brazil, though they utilize cornstarch and sugarcane juice as the main substrate for bioethanol production, which is globally seen as unsustainable because of energy, food and feed controversy (Pimental et al., 2009; Wheals et al., 1999). Bioethanol can be blended with normal gasoline in various forms: low-level blends (E10), high-level blends (E85 or E95) (Lynd, 1996). E10 (10% ethanol and 90% gasoline) is the most common ethanol blend in USA, and this can be used in new vehicle engines with non-modified. Most new cars sold in Brazil are flexible-fuel vehicles (FFV) that can run on pure 100% hydrous ethanol as well as blends with up to 80% of gasoline. In Europe, a large volume of bioethanol is used in blends with gasoline (5% ethanol and 95% gasoline) (Wheals et al., 1999). However, the market potential for bioethanol is not just limited to transport fuel or energy production but has a great potential to supply the existing chemical industry. Ethanol is also used as an oxygenate additive for conventional gasoline, as a replacement for methyl tertiary buthyl ether (MTBE), which is normally mixed with gasoline as additive to improve the octane number. Due to toxic properties associated with MTBE, which is also responsible for groundwater contamination, it is therefore more frequently replaced by ethyl tertiary butyl ether (ETBE) that is normally produced from bioethanol (Rutz and Janssen, 2007; Twidell and Weir,

2006). Ethanol is therefore an excellent additive for preventing engine knock and overheating of the engine valves (Wyman, 1996). Ethanol has higher octane number (96-113) than conventional gasoline (86-87) (Wyman, 2004) and thus, when blended the octane number increases, thereby reducing the need for toxic, octane enhancing additives (Mielenz, 2001). It enables combustion engines to run at a higher compression ratio and therefore provides a net performance gain of nearly 15% w/w (Wheals et al., 1999; Knapp et al., 1998). As earlier mentioned, the main chemical industries that patronize ethanol industry are: solvents and alcoholic for beverages.

Biogas

Biogas, a mixture of gases that is combustible when blended with air can be utilized in many ways as a source of energy (thermal, electrical or mechanical). It can also be converted to methanol and used as fuel in internal combustion engines. Biogas is mainly composed of methane (about 60% of its total content), which is colorless and odorless, flammable and lighter than air (Price and Cheremisinoff, 1981). Biogas as a combination of methane, carbon dioxide, traces of hydrogen sulfide and other elements must be purified before it can be utilized as energy source. It has many applications depending on the nature of the source and its market demand for specified energy form.

Biogas is generally used in a combined heat and power generation (CHP) plant. The utilization is mainly made up of a simultaneous production of electricity and heat for the houses. For this process, the biogas has to be drained and dried before it can be applied in a combined heat and power generation. Also, part of the heat and energy produced can be directly utilized as an internal power source in the biogas plant, thereby reducing process costs (Sommer, 2007). Along with heat, certain amount of electricity can be generated in CHP conversion and is considered as more efficient available energy extraction. Because biogas is considered as environmental-friendly and clean renewable energy, it can be upgraded and use as a vehicle fuel if purified from hydrogen sulfide. This process is needed to avoid corrosion and mechanical wear and to meet quality requirements of gas applications (Darrell, 2001).

At present, biogas is mostly used as a vehicle fuel to run the public transport in Sweden. It is also extensively utilized in other part of the world mainly in South America (Brazil) and Asia (Sommer, 2007). However, the biogas quality demands in case of vehicle engines are very strict and therefore, appropriate gas upgrading and purifying technologies must be implemented to meet the demands. These measures required that the upgraded biogas to be used in vehicle engines should contain more than 95% of pure methane without any content of carbon dioxide, water, and all the trace elements associated with biogas.

Biogas can be injected and distributed through the natural gas grid since biogas like natural gas mainly consists of methane. There are several advantages for using the gas grid to distribute biogas. One important advantage is that the grid connects the production site with more densely populated areas which enables the gas to reach new customers. It is also possible to increase the production at a remote site and still use 100% of the gas. Furthermore injecting biogas into the gas grid improves the local energy security of supply; this is an important factor for the countries that consume more gas than they produce. However, some countries in Europe like Sweden, Denmark, Germany and France have standard measures for injecting biogas into the natural gas grid. The standards have been set to avoid contamination of the gas grid or end uses. In the standards there are limits on certain components for instance sulfur, oxygen, particles and water dew point. Moreover, the electricity generated from the upgraded biogas which has been purified is usually occurring through the application of converters connected to gas generators. These converters can be in form of electrochemical cells (fuel cells) or gas turbine engines for example micro-turbine (da Costa Gomez et al., 2001; Darrell, 2001).

Biodiesel

Apart from bioethanol, other valuable liquid biofuel such as biodiesel can be integrated with production of high valuable chemicals. Clean burning mono-alkyl ester-based oxygenated fuel produced from vegetable oils, animal fats and etc. is termed biodiesel. The main substrate for biodiesel production in Europe is rapeseed oil, whereas palm oil is used for biodiesel production in Asia and Africa (Crabbe et al., 2001; Saka and Kusdiana, 2001). The extracted oil is esterified with methanol or propanol (Kiss and Bildea, 2012) generating

glycerol as its byproducts, which can be further utilized in many ways, for example to boost the biogas production or applied in chemical industry.

Because biodiesel is compatible with conventional diesel, it can be blended in any proportion with or without engine modifications. Biodiesel viscosity comes very close to that of conventional diesel and thus has no problems in the existing fuel handling system. Flash point of the biodiesel gets lowered after esterification and the octane number gets improved. Even lower concentrations of biodiesel act as octane number improver for biodiesel blends (Agarwal, 2007). More so, biodiesel blends above 20% content on vehicle engines will require some modification. Biodiesel is the main biofuel for road transport used in Europe and accounted for almost 80% percent of the biofuels market on energy basis in 2010 and Europe still the world's largest biodiesel producer, consumer, and importer (Flach et al., 2011).

High-value added chemicals

Integrated products production processes in biorefinery systems will significantly enhance its economics thereby reducing the technical and market risk. Coproducts are pillars that support biorefinery economics and increase its diversity and independency. Apart from energy products (transportation fuels, power and/or heat) from biorefinery system, other valuable material products (chemicals, food, feed etc.), which are not used for energy generation purpose can be gained from a well-designed and healthy working biorefinery system. It is therefore essential to standardize the quality requirements of biorefinery products at the starting point of this technology which will enable the system to minimize variability, as such standardization will aid focus future investigation to achieve products with specific quality (Fernando et al., 2006). In this way, products of biorefinery will be able to replace fossil materials coming from conventional oil refinery. These represent real examples of ways that a self-contained and truly integrated biorefinery system can help create a more sustainable business and enhance market value. Some important value-added chemicals have been investigated by a team from PNNL and NREL.

A list of top 12 potential biobased chemicals (fig. 8) was identified through extensive evaluating the potential markets for the building blocks and their derivatives and the

technical complexity of the synthesis pathways. These top building block chemicals and their derivatives as reported by the PNNL and NREL can be produced from monomeric sugars of lignocellulosic biomass through biological and chemical conversions, where the building blocks can be subsequently converted to a wide range of high-value biobased chemicals or materials.

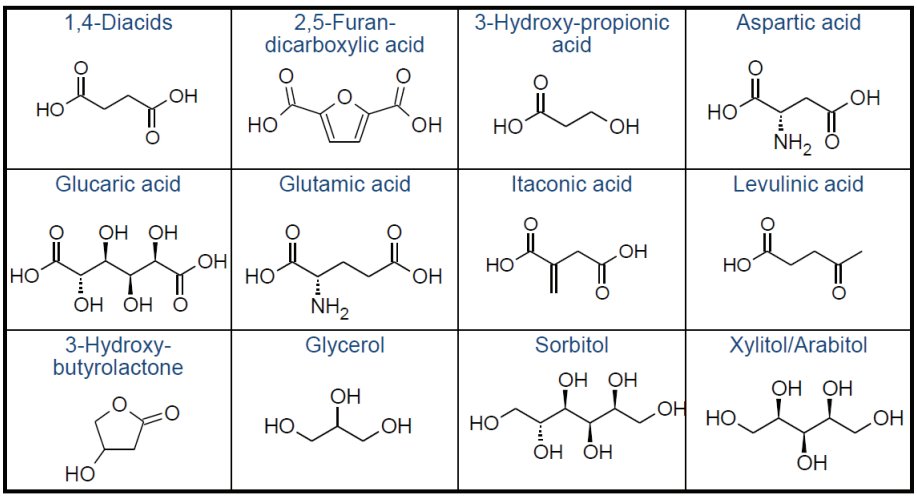

Fig. 8. Top 12 potential biobased chemicals that can be produced from biomass (Aden et al., 2004).

Building block chemicals, as considered for this analysis are molecules with multiple functional groups that possess the potential to be transformed into new families of useful molecules. The PNNL and NREL studied the synthesis for each of the top building blocks and their derivatives as a two-part pathway, where the first part is the transformation of sugars to the building blocks and the second part is the conversion of the building blocks to secondary chemicals or families of derivatives. Biological transformations account for the majority of routes from plant feedstocks to building blocks, but chemical transformations predominate in the conversion of building blocks to molecular derivatives and intermediates. The challenges and complexity of these pathways, as they relate to the use of

biomass derived sugars and chemicals, were briefly examined by a term from PNNL and NREL in order to highlight R&D needs that could help improve the economics of producing these building blocks and derivatives (Aden et al., 2004).

The use and application of these building block chemicals are huge for example, C4 dicarboxylic acids can be transformed and use as a solvents, fibers and water soluble polymers for water treatment, polyesters and nylons with new properties potentially for fiber applications can be generated from 2,5-furan dicarboxylic acid, contact lenses, diapers can be produced from 3-hydroxypropionic acid, aspartic acid can be transformed to produce salts for chelating agent and sweeteners, solvents and nylons can be generated from transformation of glucaric acid, glutamic acid can be transformed into monomers for polyesters and polyamides, products like nitrile latex or solvents can be produced from itaconic acid, levulinic acid can be transformed into fuel oxygenates and solvents, intermediate for high value pharm compounds can generated from transformation of 3-hydroxybutyrolactone, pharmaceutical and beverage products, and antifreeze etc. can be produced from glycerol transformation, sorbitol can be transformed into antifreeze and water soluble polymers, while, xylitol/arabinitol can be transformed into non-nutritive sweeteners, anhydrosugars and antifreeze. However, these products are sugar derived materials, other potential products like aromatics have a very large commodity market for polymers and surfactants, therefore, lignin a component of lignocellulosic biomass can afford the entire family of aromatic compounds that are difficult to produce through sugars (Aden et al., 2004). Carefully understanding the potential pathways and all the technical barriers associated with the transformation of these high-value marketable biobased products would lead to a better definition of which products hold the most promise as economic derivers for an integrated biorefinery system.

LCF biorefinery process economics

The conversion platform of cellulosic biomass to produce biofuels involves a complex process system, in which the production process steps takes the most of the total processing costs especially the pretreatment step, while the share of feedstock in the total costs is lower compared with the case of corn biofuels. Therefore, significant cost reductions are needed

for cellulosic biofuels production in order to make it more cost-competitive with fossil fuels for their commercial scaling-up (Carriquiry et al., 2011). It is estimated that ethanol yields from the bio-conversion of cellulosic biomass range between 110 and 300 l/t dry matter (see Table 2) (Mabee et al., 2006). Common cereal straw collectable yields of 3-5 dry t/ha and for corn stover, 4-6 dry t/ha, would result in ethanol yields per hectare varying widely between 350 to 1600 l/yr. Considering that most crop residues only have low economic value e.g. when used for animal feed, bedding, composting or heating and also often produce a problem of disposal with associated costs, cereal crops have been bred historically to reduce the straw and stover yields. Once there is a value for cellulosic biomass however, these yields per hectare could be easily increased (Sims et al., 2008). Collectable forest residue yields vary widely with tree species, age at harvest, growing conditions and many more, but when calculated on a dry t/ha per year basis, would be in a similar range to crop residues (Wright and Brown, 2007).

Table 2. Typical ethanol and energy yields recoverable from agricultural (straw, stover) or forest (wood) residues.

	Ethanol yield (liters/dry t)		Energy yields* (GJ ethanol/dry t)	
Biomass	Low	High	Low	High
Agricultural residues	110	270	2.3	5.7
Forest residues	125	300	2.6	6.3

*Based on 21.1 MJ/l ethanol lower heating value (23.6 MJ/l higher heating value). Source: Sims et al. (2008).

Considering that the energy content of cellulosic biomass is around 20 GJ/dry t, the process conversion efficiency of 1 tonne of biomass to give an energy yield of 2.3-2.6 GJ of liquid biofuels at the low end of the range is only around 12-15%. At the high end of the range, 5.7-6.3 GJ of biofuels is achievable, being closer to 35% efficiency. This reflects the theoretical maximum conversion efficiency possible based on a cellulosic biomass containing 70% carbohydrates and possibly having complete conversion of carbohydrate to ethanol with up to 51% efficiency (Sims et al., 2008; Wright and Brown, 2007). High

efficiency conversion has been successfully obtained under laboratory conditions, but it still under investigations if it will be possible under industrial conditions (Melin and Hurme, 2011). One way to improve the overall conversion process economy and to achieve the overall energy efficiency is to combust the lignin component to provide process heat, or possibly by using some of the pentose sugars to produce high value biochemical or other biomaterials.

CONCLUSIONS

The biorefinery industry is today rapidly moving forward and is favourable with increasing prices and depleting fossil oil coupled with relatively stable prices of biomass. A key driver for the development and implementation of today's biorefineries is the rapid growth in demand for renewable and sustainable biofuels and the precursor's chemicals. However, a crucial step in developing this industry is to ultimately establish integrated biorefineries capable of efficiently converting a broad range of lignocellulosic biomass into marketable and affordable products for both short- and long-term sustainability. The raw materials for the biorefineries is available in large quantities and at low costs, but the novel technologies for processing these materials at low costs in order to be more economical viable and penetrate the market are still in research and development phases. It is therefore paramount important to implement efficient technologies that can convert these raw materials at cheaper rates enabling cost effective processes in biorefinery systems.

Nevertheless, the existing biorefinery technologies needs to be developed further and validated to commercial scale level, therefore, commercialization and policy support is needed for current- and near-term opportunities to quickly grow this industry from its present base. Notwithstanding, the cost of different process steps (from biomass handling to products) in biorefinery system needs to be reduced especially the pretreatment costs, which till-date accounts for almost 40% of the whole process costs. The pretreatment method of choice should for example maximize the enzymatic convertibility of cellulose fractions and preserve the hemicellulose sugars, should be flexible in raw materials and be able to accommodate higher biomass concentration of up to 25% dry weight to ensure a

suitable ethanol concentration for the subsequent distillation process. Such a pretreatment method (wet explosion) has been tested both in lab- and pilot-scale facility. It has the potential to be applied in an integrated biorefinery industry because it combines the mechanisms of steam-explosion, wet oxidation, and dilute acid as its mode of action. It is flexible in biomass feedstocks and can operate at high dry matter concentrations.

Development of more efficient cellulases that has higher activities and stability to enable the conversion of pretreated biomass to higher yield at lower costs will significantly reduce the process costs in biorefinery industry. To that end, consolidated bioprocessing would be a method of choice, since the cellulase production, hydrolysis and fermentation is carried out in a single step, thereby reducing the operation and material expenditures that are usually required for acquisition of enzyme mixtures. Thus, this concept has the largest potential of reducing process materials, production costs, and has higher conversion efficiencies. On the other hand, the integrated biorefinery must comply with the term "carbon slaughterhouse'' where the term "waste'' does not exist, meaning that it should be able to convert almost 100% input raw material to high-value biobased products. Therefore, the production of biofuels from cellulosic materials can only be commercially viable if co-production of high-value products is established, as coproducts would help reduce market and technical risk and brings stability enabling economically viable process in biorefinery industry.

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REFERENCES

Aden, A., Bozell, J., Holladay, J., White, J., Manheim, A., 2004. Top value added chemicals from biomass. Pacific Northwest National Laboratory and National Renewable Energy Laboratory, Richland, WA.

Agarwal, A. K., 2007. Biofuels (alcohols and biodiesel) applications as fuels for internal combustion engines. Prog. Energy Combus. Sci. 33, 233-271.

Agbor, V., Cicek, N., Sparling, R., Berlin, A., Levin, D. B., 2011. Biomass pretreatment: Fundamentals toward application. Biotechnol. Advances 29, 675-685.

Ahring, B. K., Langvad, N., 2008. Sustainable low cost production of lignocellulosic bioethanol - "The carbon slaughterhouse". A process concept developed by BioGasol. Interna. Sugar Jour. 110, 184-190.

Ahring, B. K., Westermann, P., 2007. Coproduction of bioethanol with other biofuels. Adv. Biochem. Eng. Biotechnol. 108, 289-302.

Al Seadi, T., 2001. Good practice in quality management of AD residues from biogas production - Technical report. Uni. of Southern Denmark.

Ando, S., Arai, I., Kiyoto, K., Hanai, S., 1986. Identification of aromatic monomers in steam-exploded poplar and their influence on ethanol fermentation by *Saccharomyces cerevisiae*. Jour. Ferment. Technol. 64, 567-570.

Angelidaki, I., Ellegaard, L., Sørensen, A. H., Schmidt, J. E., 2002. Anaerobic processes. In: Angelidaki, I., editor. Environmental biotechnology. Institue of Environment and Resources, Technical Uni. of Denmark. AD-biogas production.

Angelidaki, I., Ahring, B. K., 1993. Thermophilic anaerobic digestion of livestock waste: the effect of ammonia. Appl. Microbiol. Biotechnol. 38, 560-564.

Bacovsky, D., Dallos, M., Wörgetter, M., 2010. Status of 2nd generation biofuels demonstration facilities. IEA Bioenergy Task 39, Report T39-P1b.

Balat, M., Balat, H., Öz, C., 2008. Progress in bioethanol processing. Progress Energy and Combustion Sci. 34, 551-573.

Ballesteros, I., Negro, M. J., Oliva, J. M., Cabanas, A., and Ballesteros, M., 2006. Ethanol produced from steam-explosion pretreated wheat straw. Appl. Biochem. Biotechnol. 129-132, 496-508.

Ballesteros, M., Oliva, J. M., Negro, M. J., Manzanares, P., Ballesteros, I., 2004. Ethanol from lignocellulosic materials by a simultaneous saccharification and fermentation process (SFS) with *Kluyveromyces marxianus* CECT 10875. Process Biochem. 39, 1843-1848.

Banerjee, S., Sen, R., Pandey, R. A., Chakrabarti, T., Satpute, D., Giri, B. S., Mudliar, S., 2009. Evaluation of wet air oxidation as a pretreatment strategy for bioethanol production from rice husk and process optimization. Biomass and Bioenergy 33, 1680-1686.

Bayer, E. A., Chanzy, H., Lamed, R., Shoham, Y., 1998. Cellulose, cellulases and cellulosomes. Curr. Opin. Struct. Biol. 8, 548-557.

Beg, Q. K., Kapoor, M., Mahajan, L., Hoondal, G. S., 2001. Microbial xylanases and their industrial application: a review. Appl. Microbiol. Biotechnol. 56, 326-338.

Bellido, C., Bolado, S., Coca, M., Lucas, S., Gonzalez-Benito, G., Garcia-Cubero, M. T., 2011. Effect of inhibitors formed during wheat straw pretreatment on ethanol fermentation by *Pichia stipitis*. Bioresource Technology 102, 10868-10874.

Benabdallah El-Hadj, T., 2006. Biodegradation of organic micropollutants in thermophilic and mesophilic anaerobic digestion of sewage sludge – A Ph.D. dissertation. Uni. of Barcelona, Spain, ISBN: 84-690-2982-7.

Berg, J. M., Tymoczko, J. L., Stryer, L., 2002. Biochemistry. W. H. Freeman and Company, New York.

Bjerre, A. B., Olesen, A. B., Fernqvist, T., Plöger, A., Schmidt, A. S., 1996. Pretreatment of wheat straw using combined wet oxidation and alkaline hydrolysis resulting in convertible cellulose and hemicellulose. Biotechnol. Bioeng. 49, 568-577.

Bobleter, O., Binder, H., Concin, R., Burtscher, E., 1981. The conversion of biomass to fuel raw material by hydrothermal pretreatment, in Energy from Biomass, Palz, W., Chartier, P., Hall, D. O., Eds. Applied Sci. Publishers: London, UK., 554-562.

Boe, K., 2006. Online monitoring and control of the biogas process – A Ph.D. dissertation. The Technical Uni. of Denmark, ISBN: 87-92855-10-1.

Bothast, R. J., Schlicher, M. A., 2005. Biotechnological processes for conversion of corn into ethanol. Appl. Microbiol. Biotechnol. 67, 19-25.

Boussaid, A., Robinson, J., Cai, Y. J., Gregg, D. J., Saddler, J. R., 1999. Fermentability of the hemicellulose-derived sugars from steam-explosion softwood (Douglas fir). Biotechnol. Bioeng. 64, 284-289.

Buchert, J., Niemella, K., Puls, J., Poutanen, K., 1990. Improvement in the fermentability of steamed hemicellulose hydrolysate by ion exclusion. Proc. Biochem. Int. 25, 176-180.

Cantarella, M., Cantarella, L., Gallifuoco, A., Spera, A., Alfani. F., 2004a. Effect of inhibitors released during steam-explosion treatment of poplar wood on subsequent enzymatic hydrolysis and SSF. Biotechnol. Prog. 20, 200-206.

Cantarella, M., Cantarella, L., Gallifuoco, A., Spera, A., and Alfani, F., 2004b. Comparison of different detoxification methods for steam-exploded poplar wood as a substrate for the bioproduction of ethanol in SHF and SSF. Process Biochemistry 39, 1533-1542.

Cardona, C. A., Sanchez, O. J., 2007. Fuel ethanol production: Process design trends and integration opportunities. Bioresource Technology. 98, 2415-2457.

Carpita, N. C., 1996. Structure and biogenesis of the cell walls of grasses. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 445-476.

Carrasco, J. E., Saiz, M. C., Navarro, A., Soriano, P., Saez, F., Martinez, J. M., 1994. Effects of dilute acid and steam explosion pretreatments on the cellulose structure and kinetics of cellulosic fraction hydrolysis by dilute acids in lignocellulosic materials. Appl. Biochem. Biotechnol. 45, 23-34.

Carriqury, M. A., Du, X., Timilsina, G, R., 2011. Second generation biofuels: Economics and policies. Energy Policy 39, 4222-4234.

Chandel, A. K., Kapoor, R. K., Singh, A., Kuhad, R. C., 2007a. Detoxification of sugarcane bagasse hydrolysate improves ethanol production by Candida shehatae NCIM 3501. Bioresource Technology 98, 1947-1950.

Chandel, A. K., Chan, E. C., Rudravaram, R., Narasu, M. L., Rao, L. V., Ravindra, P., 2007b. Economics and environmental impact of bioethanol production technologies: An appraisal. Biotechnol. Mol. Biol. Rev. 2, 14-32.

Chen, H., Qiu, W., 2010. Key technologies for bioethanol production from lignocellulose. Biotechnol. Advances 28, 556-562.

Chen, Y., Cheng, J. J., Creamer, K. S., 2008. Inhibition of anaerobic digestion process: A review. Bioresource Technology 99, 4044-4064.

Chen, T. H., Hasimoto, A. G., 1996. Effects of pH and substrate: Inoculum ratio on batch methane fermentation. Bioresource Technology 56, 179-186.

Claassen, P. A. M., van Lier, J. B., Lopez Contreras, A. M., van Niel, E. W. J., Sijtsma, L., Stams, A. J. M.,de Vries, S. S., Weusthuis, R. A., 1999. Utilisation of biomass for the supply of energy carriers. Appl. Microbiol. Biotechnol. 52, 741-755.

Clark, T. A., Mackie, K. L., 1984. Fermentation inhibitors in wood hydrolysates derived from the softwood *Pinus radiate*. Jour. Chem. Technol. Biotechnol. 34, 101-110.

Crabbe, E., Nolasco-Hipolito, C., Kobayashi, G., Sonomoto, K., Ishizaki, A., 2001. Biodiesel production from crude palm oil and evaluation of butanol extraction and fuel properties. Process Biochem. 37, 65-71.

Cullis, I. F., Saddler, J. N., Mansfield, S. D., 2004. Effect of initial moisture content and chip size on the bioconversion efficiency of softwood lignocellulosics. Biotechnol. Bioeng. 85, 413-421.

da Costa Gomez, C., Guest, C., Al Seadi, T., 2001. Biogas from AD – Bioexell training manual. Bioexell – European Biogas Centre of Excellence.

Darrell, T. M., 2001. Biogas applications for large dairy operations: Alternatives to conventional engine-generators. Cornell Cooperative Extension Assoc. of Wyoming County. Warsaw, NY.

De Bari, I., Cuma, D., Nanna, F., Braccio, G., 2004. Ethanol production in immobilized-cell bioreactors from mixed sugar syrups and enzymatic hydrolysis of steam-exploded biomass. Appl. Biochem. Biotechnol. 133-116, 539-557.

Demain, A. L., Newcomb, M., Wu, J. D., 2005. Cellulases, clostridia and ethanol. Microbiol. Mol. Biol. Rev. 69, 124-154.

Demirbas, A., 2007. Progress and recent trends in biofuels. Progress in Energy and Combustion Science 33, 1-18.

Eggeman, T., Elander, R. T., 2005. Process and economic analysis of pretreatment technologies. Bioresource Technology 96, 2019-2025.

Eklund, R., Zacchi, G., 1995. Simultaneous saccharification and fermentation of steam-pretreated willow. Enzyme Microb. Technol. 17, 255-259.

Erdei, B., Barta, Z, Sipos, B., Reczey, K., Galbe, M., Zacchi, G., 2010. Ethanol production from mixtures of wheat straw and wheat meal. Biotechnol. for Biofuels 3, 16.

Esteghlalian, A., Hashimoto, A. G., Fenske, J. J., Penner, M. H., 1997. Modeling and optimization of the dilute sulfuric acid pretreatment of corn stover, poplar and switchgrass. Bioresource Technology, 59, 129-136.

European Commission, 2005. Biomass-Green energy for Europe, Directorate-General for Research, Directorate J – Energy, ISBN: 92-894-8466-7.

Fernandez-Bolaños, J., Felizon, B., Heredia, A., Rodriguez, R., Guillen, R., Jimenez, A., 2001. Steam-explosion of olive stones: hemicellulose solubilization and enhancement of enzymatic hydrolysis of cellulose. Bioresource Technology 79, 53-61.

Fernando, S., Adhikari, S., Chandrapal, C., and Murali, N., 2006. Biorefineries: current status, challenges, and future direction. Energy & Fuels 20, 1727-1737.

Flach, B., Lieberz, S., Bendz, K., Dahlbacka, B., 2011. EU-27 Annual biofuels report. GAIN Report Number: NL1013.

Foston, M., Ragauskas, A. J., 2010. Changes in the structure of the cellulose fiber wall during dilute acid pretreatment in *populus* studied by ^{1}H and ^{2}H NMR. Energy & Fuels 24, 5677-5685.

Galbe, M., Zacchi, G., 2002. A review of the production of ethanol from softwood. Appl. Microbiol. Biotechnol. 59, 618-628.

Georgieva, T. I., Hou, X., Hilstrøm, T., Ahring, B. K., 2008. Enzymatic hydrolysis and ethanol fermentation of high dry matter wet-exploded wheat straw at low enzyme loading. Appl. Biochem. Biotechnol. 148, 35-44.

Grootjen, D. R. J., Jansen, M. L., Van der Lans, R. G. J. M., Luyben, K. C. A. M., 1991. Reactors in series for the complete conversion of glucose/xylose mixtures by *Pichia stipitis* and *Saccharomyces cerevisiae*. Enzyme Microb. Technol. 13, 828-833.

Hamelinck, C. N., Hooijdonk, V. G., Faaij, A. P. C., 2005. Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle-and long term. Biomass and Bioenergy 28, 384-410.

Hendriks, A. T. W. M., Zeeman, G., 2009. Pretreatment to enhance the digestibility of lignocellulosic biomass. Bioresource Technology 100, 10-18.

Herrera, S., 2006. Bonkers about biofuels. Nat. Biotechnol. 24, 755-760.

Herrerat, A., Tellez-Luist, S. J., Ramiret, J. A., Vazquez, M., 2003. Production of xylose from sorghum straw using hydrochloric acid. Jour. Cereal Sci. 37, 267-274.

Holm-Nielsen, J. B., Oleskowicz-Popiel, P., Al-Seadi, T., 2007. Energy crop potential for bioenergy in EU-27. 15th European Biomass Conference and Exhibition – From Market Research to Deployment, proceedings, Berlin, Germany.

Holtzapple, M., Cognata, M., Shu, Y., Henderickson, C., 1990. Inhibition of *Tricoderma reesei* cellulase by sugars and solvents. Biotechnol. Bioeng. 36, 275-287.

Huang, H-J., Ramaswamy, S., Tschirner, U. W., Ramarao, B. V., 2008. A review of separation technologies in current and future biorefineries. Separat. Purifi. Technol. 62, 1-21.

Jacobsen, S. E., Wyman, C. E., 2000. Cellulose and hemicellulose hydrolysis models for application to current and novel pretreatment processes. Appl. Biochem. Biotechnol. 84-86, 81-96.

Jeffries T. W., 2006. Engineering yeasts for xylose metabolism. Curr. Opin. Biotechnol. 17, 320-326.

Jeffries, T. W., 1982. Comparison of *Candida tropicalis* and *Pachysolen tannophilus* for conversion of xylose to ethanol. Biotechnol. Bioeng. Symp. 12, 103-110.

Jeihanipour, A., Karimi, K., Niklasson, C., Taherzadeh, M. J., 2010. A novel process for ethanol and biogas production from cellulose in blended-fibers waste textiles. Waste Manag. 30, 2504-2509.

Josefsson, T., Lennholm, H., Gellerstedt, G., 2002. Steam explosion of aspen wood. Characterisation of reaction products. Holzforschung 56, 289-297.

Joshi, B., Bhatt, M. R., Sharma, D., Joshi, J., Malla, R., Sreerama, L., 2011. Lignocellulosic ethanol production: Current practices and recent developments. Biotechnol. Mol. Biol. Rev. 6, 172-182.

Jørgensen, H., Kutter, J. P., Olsson, L., 2003. Separation and quantification of cellulases and hemicellulases by capillary electrophoresis. Anal. Biochem. 317, 85-93.

Jørgensen, H., Kristensen, J. B., Felby, C., 2007. Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. Biofuels Bioprod. Bioref. 1, 119-134.

Kamm, B., Gruber, P. R., Kamm, M., 2006. Biorefineries - Industrial processes and products. Status quo and future directions. Vol. 1, Wiley-VCH Verlag, ISBN: 3-527-31027-4.

Kamm, B., Kamm, M., 2007. Biorefineries – multi product processes. Adv. Biochem. Engin/Biotechnol. 105, 175-204.

Kamm, B., Kamm, M., 2004a. Principles of biorefineries. Appl. Microbiol. Biotechnol. 46, 137-145.

Kamm, B., Kamm, M., 2004b. Biorefinery – systems. Chem. Biochem. Eng. Q. 18, 1-6.

Kim, S, B., Lee, J. H., Oh, K. K., Lee, S. J., Lee, J. Y., Kim, J. S., Kim, S. W., 2011. Dilute acid pretreatment of barley straw and its saccharification and fermentation. Biotechnol. Bioprocess Eng. 16, 725-732.

Kim, S., Holtzapple, M, T., 2005. Lime pretreatmen and enzymatic hydrolysis of corn stover, Bioresource Technology 96, 1994-2006.

Kiss, A. A., Bildea, C. S., 2012. A review of biodiesel production by integrated reactive separation technologies. Jour. Chem. Technol. Biotechnol. 87, 861-879.

Klinke, H. B., Thomsen, A. B., Ahring, B. K., 2004. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl. Microbiol. Biotechnol. 66, 10-26.

Klinke, H. B., Ahring, B. K., Schmidt, A. S., Thomsen, A. B., 2002. Characterization of degradation products from alkaline wet oxidation of wheat straw. Bioresource Technology 82, 15-26.

Knapp, K. T., Stump, F. D., Tejada, S. B., 1998. The effect of ethanol fuel on the emissions of vehicles over a wide range of temperatures. Jour. Air Waste Manage. Assoc. 48, 646-653.

Kristensen, J. B., Thygesen, L. G., Felby, C., Jørgensen, H., and Elder, T., 2008. Cell-wall structure changes in wheat straw pretreated for bioethanol production. Biotechnol. for Biofuels 1, 1-9.

Kuhad, R. C., Singh, A., Eriksson, K. E., 1997. Microorganisms and enzymes involved in the degradation of plant fiber cell walls. Adv. Biochem. Eng./Biotechnol. 57, 45-125.

Kumar, P., Barrett, D. M., Delwiche, M. J., Stroeve, P., 2009. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuels production. Ind. Eng. Chem. Res. 48, 3713-3729.

Kurabi, A., Berlin, A., Gilkes, N., Kilburn, D., Bura, R., Robinson, J., Markov, A., Skomarovsky, A., Gusakov, A., Okunev, O., Sinitsyn, A., Gregg, D., Xie, D., Saddler, J., 2005. Enzymatic hydrolysis of steam-exploded and ethanol organosolv-pretreated Douglas-Firby novel and commercial fungal cellulases. Appl. Biochem. Biotechnol. 121, 219-230.

Larsson, S., Quintana-Sainz, A., Reimann, A., Nilvebrant, N-O., Jönsson, L. J., 2000. Influence of lignocellulose-derived aromatic compounds on oxygen-limited growth and ethanolic fermentation by Saccharomyces cerevisiae. Appl. Biochem. Biotechnol. 84-86, 617-632.

Laser, M., Schulman, D., Allen, S. G., Lichwa, J., Antal Jr., M. J., Lynd, L. R., 2002. A comparison of liquid hot water and steam pretreatments of sugar cane bagasse for bioconversion to ethanol. Bioresource Technology 81, 33-44.

Lasure, L. L., Zhang, M., 2004. Bioconversion and biorefineries of the future. Pacific Northwest National Laboratory, National Renewable Energy Laboratory.

Lavarack, B. P., Griffin, G. J., Rodman, D., 2002. The acid hydrolysis of sugarcane bagasse hemicellulose to produce xylose, arabinose, glucose and other products. Biomass and Bioenergy 23, 367-380.

Lissens, G., Thomsen, A. B., de Baere, L., Verstraete, W., Ahring, B. K., 2004. Thermal wet oxidation improves anaerobic biodegradability of raw and digested biowaste. Environ. Sci. Technol. 38, 3418-3424.

Luo, C., Brink, D. L., Blanch, H. W., 2002. Identification of potential fermentation inhibitors in conversion of hybrid poplar hydrolyzate to ethanol. Biomass and Bioenergy 22, 125-138.

Lyko, H., Deerberg, G., Weidner, E., 2009. Coupled production in biorefineries-combined use of biomass as a source of energy, fuels and materials. Jour. of Biotechnol. 142, 78-86.

Lynd, L. R., Van Zyl, W. H., McBride, J. E., Laser, M., 2005. Consolidated bioprocessing of cellulosic biomass: An update. Curr. Opin. Biotechnol. 16, 577-583.

Lynd, L. R., Weimer, P. J., Van Zyl, W. H., Pretorius, I. S., 2002. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol. Molecul. Biol. Rev. 66, 506-577.

Lynd, L. R., 1996. Overview and evaluation of fuel ethanol from cellulosic biomass: Technology, Economics, the Environment, and Policy. Annu. Rev. Energy Environ. 21, 404-465.

Mabee, W, E., Gregg, D, J., Arato, C., Berlin, A., Bura, R., Gilkes, N., Mirochnik, O., Pan, X., Pye, E. K., Saddler, J. N., 2006. Updates on softwood-to-ethanol process development. Appl. Biochem. and Biotechnol. 29, 55-70.

Mackie, K. L., Brownell, H. H., West, K. L., Saddler, J. N., 1985. Effect of sulphur dioxide and sulphuric acid on steam explosion of aspenwood. Jour. Wood Chem. Technol. 5, 405–425.

Martin, C., Klinke, H. B., Thomsen, A. B., 2007. Wet oxidation as a pretreatment method for enhancing the enzymatic convertibility of sugarcane bagasse. Enzyme and Microbial Technology 40, 426-432.

Martin, C., Thomsen, M. H., Hauggaard-Nielsen, H., Thomsen, A. B., 2008. Wet oxidation pretreatment, enzymatic hydrolysis and simultaneous saccharification and fermentation of clover-ryegrass mixtures. Bioresource Technology 99, 8777-8782.

Martin, C., Gonzalez, Y., Fernandez, T., Thomsen, A. B., 2006. Investigation of cellulose convertibility and ethanolic fermentation of sugarcane bagasse pretreated by wet oxidation and steam explosion. Jour. Chem. Technol. Biotechnol. 81, 1669-1677.

McGinnis, G. D., Wilson, W. W., Mullen, C. E., 1983. Biomass pretreatment with water and high-pressure oxygen. The wet-oxidation process. Ind. Eng. Chem. Prod. Res. Dev. 22, 352-357.

McKendry, P., 2002. Energy production from biomass (part 1): overview of biomass. Bioresource Technology 83, 37-46.

Melin, K., Hurme, M., 2011. Lignocellulosic biorefinery economic evaluation. Cellulose Chem. Technol. 45, 443-454.

Menon, V., Rao, M., 2012. Trends in bioconversion of lignocellulose: Biofuel, platform chemicals & biorefinery concept. Prog. Energy Combu. Sci. 38, 522-550.

Mes-Hartree, M., Saddler, J. N., 1983. The nature of inhibitory materials present in pretreated lignocellulosic substrates which inhibit the enzymatic hydrolysis of cellulose. Biotechnol. Lett. 5, 531-536.

Meyer, A. S., Rosgaard, L., Sørensen H. R., 2009. The minimal enzyme cocktail concept for biomass processing. Jour. Cereal Sci. 50, 337-344.

Mielenz, J. R., 2001. Ethanol production from biomass: technology and commercialization status. Curr. Opin. Microbiol. 4, 324-329.

Mok, W. S.-L., Antal Jr., M. J., 1992. Uncatalyzed solvolysis of whole biomass hemicellulose by hot compressed liquid water. Ind. Eng. Chem. Res. 31, 1157-1161.

Moniruzzaman, M., 1995. Alcohol fermentation of enzymatic hyrolysate of exploded rice straw by Pichia stipitis. World Jour. of Microbiol. Biotechnol. 11, 646-648.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M., Ladisch, M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technology 96, 673-686.

Oleskowicz-Popiel, P., Kadar, Z., Heiske, S., Klein-Marcushamer, D., Simmons, B. A., Blanch, H., W., Schmidt, J. E., 2012. Coproduction of ethanol, biogas, protein fodder and natural fertilizer in organic farming – Evaluation of a concept for a farm-scale biorefinery. Bioresource Technology 104, 440-446.

Olofsson, K., Bertilsson, M., Liden, G., 2008. A short review on SSF – an interesting process option for production from lignocellulosic feedstocks. Biotechnol for Biofuels 1, 7.

Palmqvist, E., Hahn-Hägerdal, B., 2000. Fermentation of lignocellulosic hydrolysates II: inhibitors and mechanisms of inhibition. Bioresource Technology 74, 25-33.

Palmqvist, E., Almeida, J. S., Hahn-Hägerdal, B., 1999a. Influence of furfural on anaerobic glycolytic kinetics of *Saccharomyces cerevisiae* in batch culture. Biotechnol. Bioeng. 62, 447-454.

Palmqvist, E., Grage, H., Meinander, N. Q., Hahn-Hägerdal, B., 1999b. Main and interaction effects of acetic acid, furfural and *p*-hydroxybenzoic acid on growth and ethanol productivity of yeasts. Biotechnol. Bioeng. 63, 46-55.

Palonen, H., Thomsen, A. B., Tenkanen, M., Schmidt, A. S., Viikari, L., 2004. Evaluation of wet oxidation pretreatment for enzymatic hydrolysis of softwood. Appl. Biochem. Biotechnol. 117, 1-17.

Parekh, S., Wayman, M., 1986. Fermentation of cellobiose and wood sugars to ethanol by *Candida shehatae* and *Pichia stipitis*. Biotechnol Lett. 8, 597-600.

Pedersen, M., Meyer, A. S., 2010. Lignocellulose pretreatment severity-relating pH to biomatrix opening. New Biotechnol. 27, 739-750.

Peng, F., Peng, P., Xu, F., Sun, R-C., 2012. Fractional purification and bioconversion of hemicelluloses. Biotechnol. Advan. 30, 879-903.

Petersson, A., Thomsen, M. H., Hauggaard-Nielsen, H., Thomsen, A. B., 2007. Potential bioethanol and biogas production using lignocellulosic biomass from winter rye, oilseed rape and faba bean. Biomass and Bioenergy 31, 812-819.

Pimental, D., Marklein, A., Toth, M. A., Karpoff, M. N., Paul, G. S., McCormack, R., Kyriazis, J., Krueger, T., 2009. Hum. Ecol. 37, 1-12.

Pind, P. F., Angelidaki, I., Ahring, B. K., 2003. Dynamics of the anaerobic process: effects of volatile fatty acids. Biotechnol. Bioeng. 82, 791-801.

Price, E. C., Cheremisinoff, P. N., 1981. Biogas production and utilization. Energy Technol. Series, Ann Arbor Sci. Publishers.

Rana, D., Rana, V., Ahring, B. K., 2012. Producing high sugar concentrations from loblolly pine using wet explosion pretreatment. Bioresource Technology 121, 61-67.

Reese, E. T., 1963. Advances in enzymatic hydrolysis of cellulose and related materials. Pergamon Press, New York.

Rivard, C. J., Engel, R. E., Hayward, T. K., Nagle, N. J., Hatzis, C., Philippidis, G. P., 1996. Measurement of inhibitory potential and detoxification of biomass pretreatment hydrolysate for ethanol production. Appl. Biochem. Biotechnol. 57/58, 183-191.

Ruiz, E., Cara, C., Ballesteros, M., Manzanares, P., Ballesteros, I., Castro, E., 2006. Ethanol production from pretreated olive tree wood and sunflower stalks by an SSF process. Appl. Biochem. Biotechnol. 129, 631-643.

Rutz, D., Janssen, R., 2007. Biofuel technology handbook. WIP Renewable Energies, München, Germany.

Saha, B. C., Iten, L. B., Cotta, M. A., Wu, Y. V., 2005a. Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. Process Biochem. 40, 3693-3700.

Saha, B. C., Iten, L. B., Cotta, M. A., Wu, Y. V., 2005b. Dilute acid pretreatment, enzymatic saccharification, and fermentation of rice hulls to ethanol. Biotechnol. Prog. 21, 816-822.

Saha, B C., Bothast, R. J., 1999. Pretreatment and enzymatic saccharification of corn fiber. Appl. Biochem. Biotechnol. 76, 65-77.

Saha, B C., Bothast, R. J., 1997. Enzymes in lignocellulosic biomass conversion. ACS Symp. Ser. 666, 46-56.

Saha, B. C., 2004. Lignocellulose biodegradation and applications in biotechnology. ASC Symp. Ser. 889, 2-34.

Saka, S., Kusdiana, D., 2001. Biodiesel fuel from rapeseed oil as prepared in supercritical methanol. Fuel 80, 225-231.

Sanchez, O. J., Cardona, C. A., 2008. Trends in biotechnology production of fuel ethanol from different feedstocks. Bioresource Technology 99, 5270-5295.

Sauer, M., Porro, D., Mattanovich, D., and Branduardi, P., 2008. Microbial production of organic acids: expanding the markets. Trends in Biotechnol. 26, 100-108.

Schmidt, A. S., Thomsen, A. B., 1998. Optimization of wet oxidation pretreatment of wheat straw. Bioresource Technology 64, 139-151.

Schneider, H., Wang, P. Y., Chan, Y. K., Maleszka, R., 1981. Conversion of D-xylose into ethanol by the yeast *Pachysolen tannophilus*. Biotechnol. Lett. 3,89-92.

Schwarz W. H., 2001. The cellulosome and cellulose degradation by anaerobic bacteria. Appl. Microbiol. Biotechnol. 56, 634-649.

Shallom, D., Shoham, Y., 2003. Microbial hemicellulases. Curr. Opin. Microbiol. 6, 219-228.

Sims, R., Taylor, M., Saddler, J., Mabee, W., 2008. IEA's Bioenergy Report: From 1st –to 2nd –generation biofuel technologies. An overview of current industry and RD&D activities, IEA/OECD.

Sjöström, E., 1981. Wood chemistry: fundamentals and applications. Academic Press, New York.

Slininger, P. J., Bothast, R. J., Van Cauwenberge, J. E., Kurtzman, C. P., 1982. Conversion D-xylose to ethanol by the yeast *Pachysolen tannophilus*. Biotechnol. Bioeng. 24, 371-384.

Sommer, S. G., 2007. Case studies assessment results: Environmental externalities of centralized co-digestion, in: Al Seadi, T., Ed. The future of biogas in Europe III, conference proceedings. Uni. of Southern Denmark.

Stepan, A. M, Höije, A., Schols, H. A., de Waard, P., and Gatenholm, P., 2012. Arabinose content af arabinoxylans contributes to flexibility of acetylated arabinoxylan films. Jour. Appl. Poly. Sci. 125, 2348-2355.

Sun, X. F., Xu, F., Sun, R. C., Wang, Y. X., Fowler, P., Baird, M. S., 2004. Characteristics of degraded lignins obtained from steam exploded wheat straw. Polym. Degrad. Stabil. 86, 245-256.

Sun, Y., Cheng, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresource Technology 83, 1-11.

Sørensen, A., Teller, P. J., Hilstrøm, T., Ahring, B. K., 2008. Hydrolysis of miscanthus for bioethanol production using dilute acid presoaking combined with wet explosion pre-treatment and enzymatic treatment. Bioresource Technology 99, 6602-6607.

Taherzadeh, M. J., Karimi, K., 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production. Int. Jour. Mol. Sci. 9, 1621-1651.

Taherzadeh, M. J., Karimi, K., 2007a. Acid-based hydrolysis processes for ethanol from lignocellulosic materials: a review. BioResources 2, 472-499.

Taherzadeh, M. J., Karimi, K., 2007b. Enzyme-based hydrolysis processes for ethanol from lignocellulosic materials: a revew. BioResources 2, 707-738.

Taherzadeh, M. J., Niklasson, C., 2004. Ethanol from lignocellulosic materials: pretreatment, acid and enzymatic hydrolysis, and fermentation. ACS Symp. Ser. 889, 49-68.

Taherzadeh, M. J., Gustafsson, L., Liden, S., 2000. Physiological effects of 5-hydroxymethlfurfural on *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 53, 701-708.

Taherzadeh, M. J., Niklasson, C., Liden, G., 1997. Acetic acid-friend or foe in anaerobic batch conversion of glucose to ethanol by *Saccharomyces cerevisiae*?. Chem. Eng. Sci. 52, 2653-2659.

Taniguchi, M., Tohma, T., Itaya, T., and Fujii, M., 1997. Ethanol production from a mixture of glucose and xylose by co-culture of Pichia stipitis and a respiratory-deficient mutant of Saccharomyces cerevisiae. Jour. Ferment. Bioeng. 83, 364-370.

Thomsen, M. H., 2005. Complex media from processing of agricultural crops for microbial fermentation. Appl. Microbiol. Biotechnol. 68, 598-606.

Twidell, J., Weir, T., 2006. Renewable energy resources. Taylor and Francis, New York, USA.

Uellendahl, H., Ahring, B. K., 2010. Anaerobic digestion as final step of a cellulosic ethanol biorefinery: Biogas production from fermentation effluent in a UASB reactor – pilot-scale results. Biotechnol. Bioeng. 107, 59-64.

Van Dyk, J. S., Pletschke, B. I., 2012. A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes-Factor affecting enzymes, conversion and synergy. Biotechnol Adv. doi: 10.1016/j.biotechadv.2012.03.002.

Van Dyne, D. L., Blasé, M. L., Clements, L. D., 1999. A strategy for returning agriculture and rural America to long-term full employment using biomass refineries. In: Janeck, J., (ed) Perspectives on new crops and new uses. ASHS Press, Alexandria, VA, pp 114-123.

Vane, L. M., 2005. A review of pervaporation for product recovery from biomass fermentation processes. Jour. Chem. Technol. Biotechnol. 80, 603-629.

Van Lier, J. B., 1995. Thermophilic anaerobic waste water treatment, temperature aspects and process stability – Ph.D. dissertation. Wageningen Agricultural Uni, The Netherlands, ISBN 90-5485-436-7.

Van Zyl, W. H., Lynd, L. R., den Haan, R., McBride, J. E., 2007. Consolidated bioprocessing for bioethanol production using *Saccharomyces cerevisiae*. Adv. Biochem. Engin./Biotechnol. 108, 205-235.

Varga, E., Reczey, K., Zacchi, G., 2004. Optimization of steam pretreatment of corn stover to enhance enzymatic digestibility. Appl. Biochem. Biotechnol. 113, 509-523.

Varga, E., Schmidt, A. S., Reczey, K., Thomsen, A. B., 2003. Pretreatment of corn stover using Wet Oxidation to enhance enzymatic digestibility. Appl. Biochem. and Biotechnol. 104, 0273-2289.

Vazquez, M., Oliva, M., Tellez-Luis, S. J., Ramirez, J. A., 2007.Hydrolysis of sorghum straw using phosphoric acid: Evaluation of furfural production. Bioresource Technology 98, 3053-3060.

Ward, O. P., Moo-Young, M., 1989. Enzymatic degradation of cell wall and related plant polysaccharides. CRC Crit. Rev. Biotechnol. 8, 237-274.

Weil, J. R., Dien, B., Bothast, R., Hendrickson, R., Mosier, N. S., Ladisch, M. R., 2002. Removal of fermentation inhibitors formed during pretreatment of biomass by polymeric adsorbents. Ind. Eng. Chem. Res. 41, 6132-6138.

Westermann, P., Ahring, B. K., 2005. The biorefinery for production of multiple biofuels. In: Lens, P., Westermann, P., Haberbauer, M., Moreno, A., editors. Biofuels for fuels cells: Renewable energy from biomass fermentation. London, UK: IWA Publishing, p 194-205.

Wheals, A. E., Basso, L. C., Alves, D. M. G., Amorim, H. V., 1999. Fuel ethanol after 25 years. Trends Biotechnol. 17, 482-487.

Wilson, D. B., 2011. Microbial diversity of cellulose hydrolysis. Curr. Opin. Microbiol. 14, 259-263.

Wilson, D. B., 2009. Cellulases and biofuels. Curr. Opin. Biotechnol. 20, 295-299.

Wright, M. M., Brown, R. C., 2007. Comparative economics of biorefineries based on the biochemical and thermochemical platforms. Biofuels Bioprod. Bioref. 1, 49-56.

Wyman, C. E., Decker, S. R., Himmel, M. E., Brady, J. W., Skopec, C. E., Viikari, L., 2005a. Hydrolysis of cellulose and hemicellulose. Polysaccharides: structural diversity and functional versatility. S. Dumitriu, Ed. Marcel Dekker INC., New York, 2nd edn, 995-1033.

Wyman, C. E., Dale, B. E., Elander, R. T., Holtzapple, M., Ladisch, M. R., Lee, Y. Y., Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover. Bioresource Technology 96, 2026-2032.

Wyman, C. E., 1994. Ethanol from lignocellulosic biomass: Technology, Economics, and Opportunities. Bioresource Technology 50, 3-16.

Wyman, C. E., 1996. Handbook on bioethanol: production and utilization. Taylor & Francis: Washington DC, USA.

Xiros, C., Vafiadi, C., Paschos, T., Christakopoulos, P., 2010. Toxicity tolerance of *Fusarium oxysporum* towards inhibitory compunds formed during pretreatment of lignocellulosic materials. Jour. Chem. Technol. Biotechnol. 86, 223-230.

Zaldivar, J., Nielsen, J., Olsson, L., 2001. Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. Appl. Microbiol. Biotechnol. 56, 17-34.

Zhang, Y. H., Berson, E., Sarkanen, S., Dale, B. E., 2009. Pretreatment and biomass recalcitrance: Fundamentals and Progress. Appl. Biochem. Biotechnol. 153, 80-83.

Zheng, Y., Pan, Z., Zhang, R., 2009. Overview of biomass pretreatment for cellulosic ethanol production. Int. Jour. Biol. Eng. 2, 51-68.

Zhu, J. J., Yong, Q., Xu, Y., Chen, S. X., Yu, S. Y., 2009. Adaptation fermentation of Pichia stipitis and combination detoxification on steam exploded lignocellulosic prehydrolyzate. Nat. Sci. 1, 47-54.

Research paper I

Pretreatment as the crucial step for cellulosic biorefinery: Testing the efficiency of wet explosion on different types of biomass

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Pretreatment as the crucial step for a cellulosic ethanol biorefinery: Testing the efficiency of wet explosion on different types of biomass

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Abstract

The efficiency of wet explosion applied as modified dilute acid pretreatment at previously identified reference conditions (150 °C, 0.3% H_2SO_4, 15 min) was investigated on lucerne, ryegrass, fescue grass, cocksfoot grass, rye fescue, forage grass, and wheat straw in order to identify their potential as feedstock for cellulosic bioethanol production.

After pretreatment, cellulose recovery was more than 95% for all biomass while enzymatic convertibility of cellulose ranged from 40% to 80%. Lower enzymatic conversion of cellulose was correlated with higher lignin content of the biomass. Hemicellulose recovery was 81-91% with a final pentose yield of 65-85%. Cocksfoot grass and wheat straw had the highest bioethanol potential of 292 and 308 L/ton DM, respectively. Overall efficiencies were higher than 68% for cocksfoot grass harvested in August, fescue grass, wheat straw, and forage grass while efficiencies were lower than 61% for the other tested biomass resources, making further adjustment of the process parameters necessary.

Keywords: Wet explosion; Lignocellulosic biomass; Pretreatment; Enzymatic hydrolysis; Bioethanol

1. INTRODUCTION

Lignocellulosic plant biomass is regarded as potential feedstock for sustainable production of biobased products including biofuels. However, due to the resistant of lignocellulosic structure, the utilization of cellulosic sugars to produce valuable biobased products faces significant technical challenges and its success depends largely upon the physical and chemical properties of the biomass type and pretreatment methods applied to it (Himmel et al., 1997). Pretreatment is among the most costly steps in the biochemical conversion of lignocellulosic biomass, accounting for up to 40% of the total processing cost (Lynd, 1996; Percival Zhang et al., 2009). Various thermal and chemical pretreatment methods as well as combinations of both have been proposed to make lignocellulosic biomass susceptible to enzymatic and microbial conversion (Galbe and Zacchi, 2002, Hendriks and Zeeman, 2009). Effective pretreatment of lignocellulosic biomass is characterized by a reduction in particle size, increase in surface area (porosity), disruption of cellulose crystallinity, hemicellulose disruption, and lignin redistribution without the formation of degradation products that inhibit the microbial activities during ethanol fermentation (Mosier et al., 2005; Karimi et al., 2006).

Wet explosion (WEx) has previously been shown to be very effective for the pretreatment of wheat straw and Miscanthus (Georgieva et al., 2008; Sørensen et al., 2008) and has been applied in pilot-scale as part of the so-called MaxiFuel concept (Westermann et al., 2005; Ahring and Westermann, 2007) for bioethanol production from lignocellulosic biomass. This biorefinery concept includes the conversion of both C6 and C5 sugars into ethanol, the production of biogas from the fermentation effluent, and the separation of a lignin fraction that can be pelletized for combustion or converted into other valuable products.

The aim of the current study was to screen different types of local biomass from the island of Bornholm, Denmark, for their potential of bioethanol production in a projected demonstration plant for this concept. The results of the screening will form the basis for the selection of two types of biomass with the highest potential for further optimization of process parameters of the different steps of the whole concept. The previously developed

reference parameter set for the modified WEx pretreatment of wheat straw with dilute acid addition was used for the screening of the pretreatment on the local biomass resources lucerne, ryegrass, fescue grass, cocksfoot grass, rye fescue, forage grass, and wheat straw. Subsequent enzymatic hydrolysis of cellulose was performed to investigate the efficiency of wet explosion for the final sugar release from the selected biomass as basis for their theoretical ethanol production.

2. MATERIAL AND METHODS

2.1. Biomass samples

Ten different samples of seven different grass and straw species, Lucerne, *Medicago sativa* (Marshal), ryegrass, *Lolium* (Mathilde), fescue grass, *Festuca arundinacea* (Hykor), cocksfoot grass, *Dactylis glomerata*, (Amba), rye fescue, *Festulolium* (Perun), forage grass - a mixture of 10% red clover grass (Rajah), 10% white clover grass (Klondike), 40% rye fescue (Perun), 20% ryegrass (Indiana), 20% ryegrass (Mikado), and wheat straw were collected from Bornholm, Denmark. The biomass samples represented the main species available on Bornholm and were collected according to their typical harvest times in order to evaluate their potential in the current agricultural practice on Bornholm. Forage grass was collected in July and November, fescue grass in October, wheat straw in August, cocksfoot grass in August and November, lucerne in October and November, and ryegrass and rye fescue in November. All samples were air-dried and hammer milled to a particle size of 2-3 mm and stored in plastic bags at room temperature prior to pretreatment. Dry matter content for all dried biomass samples ranged from 84 to 93 g DM/100 g material.

For chemical composition analysis, a portion of each raw biomass sample was ground in a coffee grinder to a particle size of 1 mm. The content of total carbohydrates (cellulose and hemicellulose), and Klason lignin in the raw biomass was determined by strong acid hydrolysis according to the procedure developed by the National Renewable Energy Laboratory (Sluiter et al., 2008a).

Sugar analysis (glucose and xylose) was performed by high performance liquid chromatography (HPLC) with refractive index (RI) detection (Shimadzu Corp., Japan) on

an Aminex HPX-87H column (Bio-Rad Laboratories, CA, USA) using 4 mM H_2SO_4 as eluent and a flow rate of 0.6 mL/min at 60 ^oC**.** Prior to HPLC analysis, samples were centrifuged at 4000 *g* for 10 min, and filtered through a 0.45 µm syringe filter.

Dry matter content (DM), volatile solid content (VS), and ash were determined according to the procedure described by the American Public Health Association (APHA, 1992).

2.2. Wet explosion pretreatment

The wet explosion pretreatment was performed in a 5 L WEx reactor, equipped with a high-pressure cylinder, a gas/liquid inlet for injection of dilute sulfuric acid, and a continuous stirrer (990 rpm). The reactor was heated by a water jacket connected to a heat exchanger controlled by an oil heater. The temperature inside the reactor was monitored by a temperature sensor mounted in the headspace. The pretreatment of all biomass samples was performed at previously identified reference conditions (150 ^oC, 15 min retention time) by suspending 150 g dried biomass in 820 g of tap water. A final acid concentration of 0.3% and a dry matter content of 12-13% was reached by injection of 30 g of 10% sulfuric acid into the WEx reactor after reaching the desired temperature.

After the treatment, the biomass was flashed into a 20 L flash tank connected to the reactor, resulting in a sudden drop in temperature and pressure.

The resulting biomass slurry from the pretreatment was separated into liquid and solid fractions. The separation was performed in a commercial filtration unit (Larox Buchner unit) with a filtering cloth pore size of 20 µm and a constant vacuum pressure of -0.7 psi. The solid fraction was washed thoroughly with water and stored in a freezer (-18^oC) prior to compositional analysis and further processing. The separated liquid fraction was stored at 4 ^oC before further analyses.

2.3. Analysis of the solid fraction

A part of the solid fraction (washed filter cake) of the WEx slurry was dried in an incubator at 38 ^oC for 12 h and ground in a coffee grinder to a 1 mm particle size before composition analysis. The content of total carbohydrates (cellulose, hemicellulose), and

Klason lignin in the separated solid fractions, Cell $_{SF}$, Hemicell $_{SF}$ and Lignin $_{SF}$ was determined by strong acid hydrolysis as previously described (Sluiter et al., 2008a).

The recovery of cellulose and hemicellulose during the WEx process was calculated by using Eq. (A.1) and (A.2):

$$\text{Cellulose}(\%) = \frac{\text{Cell}_{SF} + \text{Glucose}_{LF}/1.11}{\text{Cell}_{RB}} \cdot 100 \qquad \text{(A.1)}$$

$$\text{Hemicellulose}\ (\%) = \frac{\text{Hemicell}_{SF} + (\text{Xylose}_{LF} + \text{Arabinose}_{LF})/1.14}{\text{Hemicell}_{RB}} \cdot 100 \qquad \text{(A.2)}$$

where Glucose $_{LF}$ is the glucose in the liquid fraction, Cell $_{SF}$ and Cell $_{RB}$ are the cellulose, and Hemicell $_{SF}$ and Hemicell $_{RB}$ are the hemicellulose found in the solid fraction, and in the raw biomass, respectively, while Xylose $_{LF}$ and Arabinose $_{LF}$ are the xylose and arabinose in the liquid fraction, respectively; 1.11, and 1.14 are the stoichiometric conversion factors of polysaccharides to sugar monomers.

2.4. Enzymatic hydrolysis of solid fraction

The release of hydrolysable cellulose by the wet explosion pretreatment was analyzed by the sugars released after enzymatic hydrolysis using a commercial enzyme mixture (Cellic CTec), kindly provided by Novozymes A/S (Bagsværd, Denmark). The enzymatic conversion of the separated wet exploded solid fraction was carried out at 5% DM with 0.05 M succinate buffer (pH 5.0). The experiments were performed in duplicates in 2 mL Eppendorf tubes filled with 1.5 mL of hydrolysis media and at an enzyme dosage of 20 mg-EP/g-VS (EP = enzyme protein) for all samples. The hydrolysis mixture was incubated for 72 h at 50 ^{o}C in a thermomixer shaker at 1400 rpm. The reaction was stopped by heating the solution to 100 ^{o}C for 10 min, mixed by vortexing, and centrifuged for 8 min at 3600 *g*. The concentration of glucose, xylose, and arabinose in the hydrolyzate was quantified by HPLC as described in Section 2.1.

The glucose yield after enzymatic hydrolysis was calculated according to Eq. (B.1):

$$\text{Hydrolysis yield}_{\text{glucose}}\ (\%) = \frac{\text{Glucose}_{\text{EH}}\ / 1.11}{\text{Cell}_{\text{RB}}} \cdot 100 \qquad \text{(B.1)}$$

where Glucose $_{EH}$ is the mass of glucose released after enzymatic hydrolysis of cellulose in the solid fraction.

2.5. Analysis of the liquid fraction

The concentration of sugar monomers (glucose, xylose, arabinose), 2-furfural, hydroxymethylfurfural (HMF), and carboxylic acids (acetic acid and glycolic acid) present in the liquid fraction (filtrate) after WEx pretreatment were directly quantified by HPLC analysis as described in Section 2.1 without further treatment. While the total soluble sugars present as oligomers in the liquid fractions were hydrolyzed with 4% w/w sulfuric acid at 121 ^{o}C for 10 min, and determined according to the National Renewable Energy Laboratory protocol (Sluiter et al., 2008b). The yield of pentose sugars after WEx pretreatment was calculated according to Eq. (B.2):

$$\text{Hydrolysis yield}_{\text{pentoses}}(\%) = \frac{\text{Pentoses}_{\text{LF}} / 1.14}{\text{Hemicell}_{\text{RB}}} \cdot 100 \qquad \text{(B.2)}$$

where Pentoses $_{LF}$ is the mass of the pentose sugars xylose and arabinose released after WEx pretreatment in the liquid fraction.

The theoretical ethanol production was calculated based on the total yield of C6 and C5 sugars after WEx pretreatment and enzymatic hydrolysis of cellulose fraction according to Eq. (C.1):

$$\text{Yield}_{\text{EtOH,tot}} = \text{Glucose}_{\text{tot}} \times 0.51 + \text{Pentose}_{\text{tot}} \times 0.50 \qquad \text{(C.1)}$$

where Glucose $_{tot}$ is the total glucose released after pretreatment and enzymatic hydrolysis and Pentoses $_{tot}$ is the total pentoses released from hemicellulose after WEx pretreatment and 0.51 and 0.50 are the maximal achievable ethanol fermentation yields (in g-EtOH/g-sugar) from glucose and xylose, respectively (Hatzis et al., 1996).

3. RESULTS AND DISCUSSION

3.1. Raw biomass composition

The composition of the raw biomass is shown in Fig. 1. Cellulose content of the raw biomass was highest in wheat straw (collected in August), lucerne harvested in October, and cocksfoot grass harvested in August (35.6–36.5 g/100 g DM). For all other biomass samples, the cellulose content was below 32 g/100 g DM. The highest hemicellulose content (23.4 g/100 g DM) was found in cocksfoot grass harvested in August followed by wheat straw with similar hemicellulose content (23.3 g/100 g DM. Accordingly, the sum of cellulose and hemicellulose was highest for wheat straw and cocksfoot grass harvested in August. These values are higher than the values found for wheat straw by Thomsen et al. (2006) (30.4 g/100 g DM for cellulose and 21.3 g/100 g DM for hemicellulose).

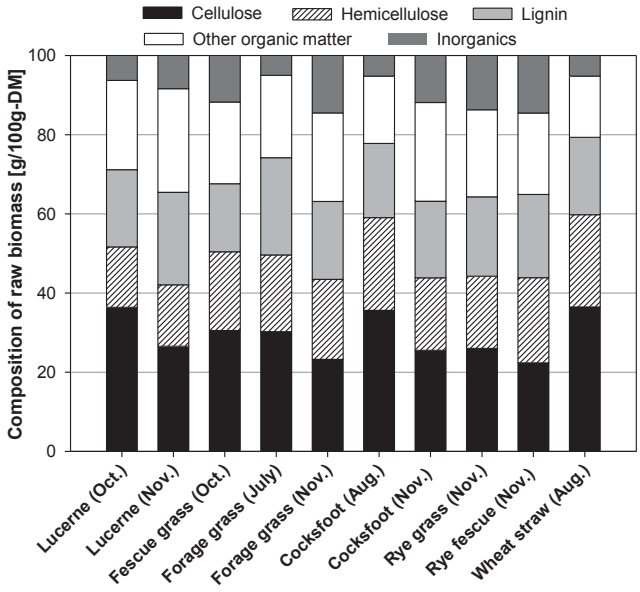

Fig. 1. Chemical composition of the different biomass samples.

The lignin content ranged between 17.2 and 24.6 g/100g DM with the highest value for forage grass harvested in July. Comparing the composition of the biomass for the different harvest times reveals a general pattern that the cellulose content decreases and the content of inorganics increases with later harvest of the biomass. For lignin and hemicellulose, however, there was no clear correlation. For lucerne, the lignin content was higher for the biomass harvested later, while it is lower for forage grass and about the same for cocksfoot grass. The hemicellulose content remained about the same for lucerne and forage grass while it was significantly lower for cocksfoot grass harvested in November (Fig. 1). The content of other organic matter, which is the sum of non-analyzed organic matter like pentoses other than xylose and arabinose, proteins and fats, was also not clearly correlated with harvest time as it was higher for lucerne and cocksfoot grass at a later harvest time and remained the same for forage grass harvested in November (Fig. 1).

3.2.WEx pretreatment and recovery of carbohydrates and lignin

WEx pretreatment with dilute acid addition was expected to fractionate the lignocellulosic biomass into a solid fraction containing mainly cellulose and lignin, and a liquid fraction enriched with solubilized hemicelluloses, mainly present as C5 monomers, and low molecular lignin fragments in dissolved form.

The recovery of cellulose in the solid fraction was high for all biomass samples, and ranged from 95 to 99%, while the recovery of hemicellulose was within the range of 81-91% (Fig. 2). The highest cellulose recovery of approximately 99% was achieved with ryegrass harvested in November and forage grass harvested in July. The recoveries of hemicellulose were generally above 89%, except for wheat straw and cocksfoot grass harvested in August that gave hemicellulose recoveries of 81 and 82%, respectively.

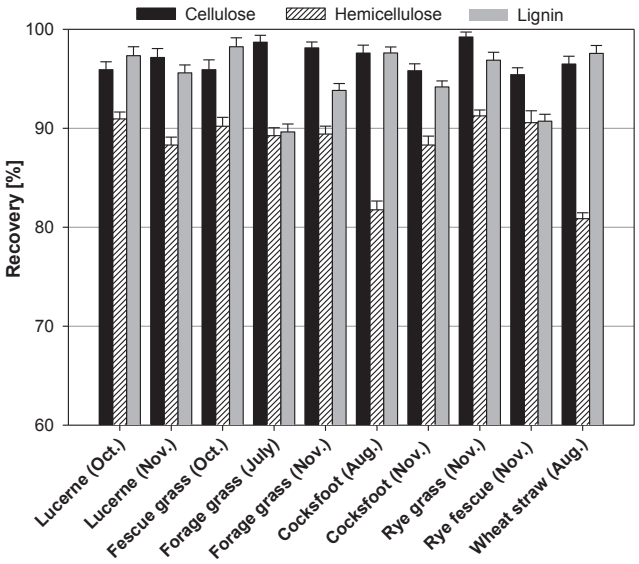

Fig. 2. Recovery of polysaccharides (cellulose and hemicellulose) and lignin after modified WEx pretreatment at reference conditions for the different biomass samples. (error bars represent standard deviations of duplicates).

The recoveries for wheat straw (96% cellulose and 81% hemicellulose) are in good agreement with those of Georgieva et al. (2008), who reported that 93% of cellulose and 72% of hemicellulose were found after wet explosion of wheat straw at 185 ^{o}C for 15 min and 35% (v/v) hydrogen peroxide. In comparison, Martin et al. (2007) achieved 70% cellulose recovery after treating sugarcane bagasse by wet oxidation at 195 ^{o}C, 15 min and alkaline pH. The high recovery of cellulose among the biomass samples at the lower temperature applied in the current study reveals an efficient separation of the cellulose from lignin and low degradation of cellulose to other products such as HMF during the WEx process. The relatively high hemicellulose recoveries compared to those from other studies indicate relatively low sugar degradation during the dilute acid WEx pretreatment at the applied lower temperature.

Lignin recoveries in the solid fraction were more than 89% for all treated biomass samples (Fig. 2). High lignin recoveries above 97% were found in lucerne harvested in

October, fescue grass, cocksfoot grass harvested in August, and wheat straw, while recoveries of around 90% were found for forage grass harvested in July and rye fescue. As lignin recovery in the solid fraction after pretreatment is affected by solubilization and depolymerization of sugars (Sassner et al., 2008), and lignin may be degraded to low molecular lignin compounds i.e. phenolic compounds under harsh pretreatment conditions (Petersen et al., 2009), the high recoveries of lignin in the solid fraction indicate that only a small part of the lignin was solubilized and, hence, the formation of low molecular lignin compounds was low.

3.3. Enzymatic hydrolysis of cellulose in the solid fraction and yield sugars in the liquid fraction after pretreatment

The enzymatic convertibility of cellulose under the tested WEx conditions is one of the most important factors to evaluate the efficiency of the pretreatment for the production of bioethanol from C6 sugars (Varga et al., 2003) as it reveals the efficiency of enzymatic hydrolysis specifically for a certain enzyme mixture on the specific hydrolyzate.

Fig. 3A depicts the yields of glucose and pentoses after enzymatic hydrolysis of the wet-exploded solid fraction (filter cake). Wheat straw and cocksfoot grass harvested in August gave the highest glucose yields of 80% and 74%, respectively. In comparison, Thomsen et al. (2006) reported only 67-68% glucose yield from the solid fraction after hydrothermal pretreatment of wheat straw at 190 ^{o}C to 200 ^{o}C. The lowest glucose yield of 40-54% was found with lucerne although this biomass gave a good cellulose recovery. These results indicate that enzymatic convertibility of cellulose to sugar monomers was the main bottleneck for biomass like forage grass harvested in July and lucerne, which is obviously correlated to the higher lignin content found in these biomass samples (24.6 and 23.4 g/100 g DM, respectively). These findings are in agreement with the proposition that high lignin content of biomass blocks enzyme accessibility, causes end-product inhibition, and reduces the rate and yield of enzymatic hydrolysis (Knauf and Moniruzzaman, 2004; Yang et al., 2009). The solubilized pentose sugars (xylose and arabinose) were mainly dissolved in the liquid fraction and only 10-19% was found in the solid fraction (Fig. 3A). The pentose yields in the liquid fraction were above 70% for most of the biomass samples apart from

lucerne, cocksfoot grass and wheat straw harvested in November and August, with a pentose yield slightly below 70% (Fig. 3B).

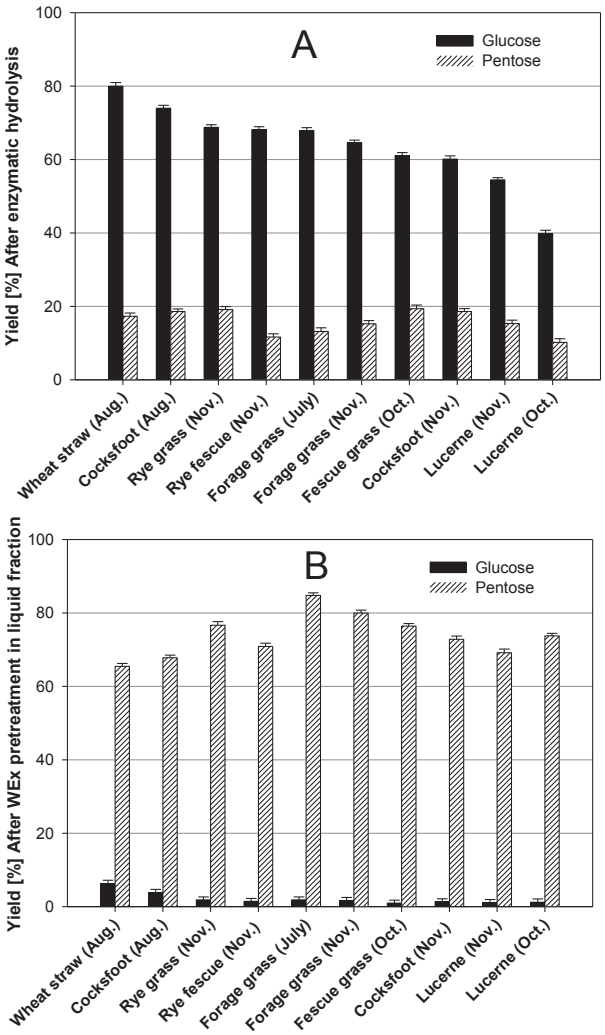

Fig. 3(A). Yield of glucose and pentoses after enzymatic hydrolysis of solid fractions. **(B).** Yield of glucose and pentoses in liquid fraction after modified WEx pretreatment. Average values and standard deviations reported for duplicates.

The highest pentose yield of approximately 85% was achieved with forage grass harvested in July. In comparison, Lu et al. (2009) found a xylose yield of 70% for 0.5-2% sulfuric acid-catalyzed hydrothermal pretreatment (180 ^{o}C, 5-20 min) of rapeseed straw. These results show that hemicellulose sugars were, for the most part, extracted and released into the liquid fraction during the dilute acid pretreatment. The total pentose yields in the liquid and solid fraction after WEx pretreatment and enzymatic hydrolysis were generally above 80%, except for cocksfoot grass harvested in August, lucerne harvested in October, and rye fescue harvested in November. The glucose yield in the liquid fraction was low compared to the pentose yields for all treated biomass types, indicating that only a very small fraction of glucose monomers originating from cellulose was solubilized during pretreatment.

Overall, these results show that the severity of the applied WEx treatment at 150 ^{o}C with a low acid dosage was for most of the tested biomass high enough to release a high amount of glucose from the cellulose and mild enough not to convert the pentose sugars released from the hemicellulose into undesired degradation products.

3.4. Formation of degradation products during WEx pretreatment

The concentration of carboxylic acids, furfural and HMF in the liquid fraction after pretreatment is presented in Table 1. Depending on process severity, carboxylic acids, furan derivatives (furfural and 5-hydroxymethyl furfural-HMF), and phenolic compounds were generated during pretreatment of lignocellulosic biomass. These products are considered potential fermentation inhibitors (Saha, 2004; Klinke et al., 2004). At more severe pretreatment conditions, xylose is degraded to furfural while HMF is formed from hexose degradation, and phenolic compounds are generated from partial breakdown of lignin (Palmqvist and Hahn-Hägerdal, 2000). Carboxylic acids were analyzed as the sum of acetic and glycolic acids. The highest by-product formation was found with cocksfoot grass (August harvest) and wheat straw at levels of 0.9 and 1.5 g/100 g DM for carboxylic acids, 0.2 and 0.3 g/100 g DM for furfural, and below 0.1 g/100 g DM for HMF, respectively. For all other biomass samples, the concentrations were significantly lower. In comparison, Lu et al. (2009), performing sulfuric acid-catalyzed hydrothermal pretreatment at 0.5-2% acid

concentration, 5-20 min, and 180 ^{o}C, found 4.1 g/100 g DM for acetic acid and 1.1 g/100 g DM for furfural and HMF.

Table 1. Formation of by-products (g/100g DM) in the liquid fraction after WEx pretreatment of the different biomass samples.

Biomass	Carboxylic acids[a]	Furfural	HMF
	g/100g DM	g/100g DM	g/100g DM
Wheat straw (Aug.)	1.53 (0.04)	0.25 (0.02)	0.03 (0.00)
Cocksfoot (Aug.)	0.93 (0.01)	0.14 (0.00)	0.10 (0.00)
Rye grass (Nov.)	0.15 (0.02)	0.07 (0.00)	0.03 (0.00)
Rye fescue (Nov.)	0.08 (0.03)	0.05 (0.02)	0.02 (0.00)
Forage grass (July)	0.10 (0.01)	0.05 (0.01)	0.02 (0.00)
Forage grass (Nov.)	0.09 (0.02)	0.06 (0.03)	0.02 (0.00)
Fescue grass (Oct.)	0.08 (0.00)	0.02 (0.00)	0.01 (0.00)
Cocksfoot (Nov.)	0.18 (0.05)	0.01 (0.00)	0.00 (0.00)
Lucerne (Nov.)	0.09 (0.01)	0.03 (0.00)	0.01 (0.00)
Lucerne (Oct.)	0.15 (0.03)	0.01 (0.00)	0.00 (0.00)

Average of duplicates. Standard deviation shown in parentheses. [a]Sum of acetic and glycolic acids.

The formation of by-products in the current study was also lower compared to that found by Martin et al. (2007) who found 9.21 g/100 g material for carboxylic acids, 0.53 g/100 g material for furfural, and 0.07 g/100 g material for HMF after wet oxidation pretreatment of sugarcane bagasse at 195 ^{o}C, 15 min at alkaline pH. The lower by-product formation in the current study indicates that the severity of the pretreatment used in the current study is significantly lower compared to the abovementioned studies, mainly due to the lower temperature.

3.5. Theoretical ethanol production

The maximum achievable ethanol yield was calculated for the different biomass samples based on the total yields of C6 and C5 sugars after WEx pretreatment and enzymatic hydrolysis of cellulose. Since the current study should not be committed to a certain fermentation design, the calculated ethanol yield was based on the maximum yield of 0.51 g-EtOH/g-glucose and 0.50 g-EtOH/g-xylose, as reported by Hatzis et al. (1996). Due to the same pretreatment conditions used and the relatively low production of furfural and HMF, it was expected that the efficiency of the fermentation would not vary significantly for the different pretreated biomass resources.

Using the sugar yields achieved after WEx pretreatment and enzymatic hydrolysis, the calculated ethanol yields among the different pretreated types of biomass ranged from 177 to 308 L/ton DM (Fig. 4). The ratio of ethanol yield based on the sugar release after WEx pretreatment and enzymatic hydrolysis and the potential ethanol yield based on the composition of the raw biomass is an expression for the efficiency of the applied WEx treatment. As can be seen in Fig. 4, this ratio, i.e. the pretreatment efficiency, was quite different for the different biomass samples, with values higher than 68% for cocksfoot grass harvested in August, fescue grass, wheat straw, and forage grass and lower than 61% for the other tested biomass resources. However, no correlation was found between biomass composition (content of cellulose, hemicellulose or lignin) and efficiency of the pretreatment (data not shown). The highest achievable ethanol yields of 292 and 308 L/ton DM could be obtained from cocksfoot grass harvested in August and wheat straw, respectively, corresponding to a yield of 74% and 80% of the potential based on the cellulose content of the biomass, and a yield of 59% and 61% of the potential based on the hemicellulose content.

For all other biomass samples, the potential and maximum achievable ethanol yields were significantly lower. For forage grass harvested in July and fescue grass, the pretreatment efficiency under the tested conditions was, however, the highest among all samples, leading to ethanol yields of up to 250 L/ton DM. For all other samples the maximum achievable ethanol yield was around 200 L/ton DM with the lowest for lucerne harvested in October. For this biomass the efficiency of the pretreatment was less than

50%, which was mainly due to the low enzymatic convertibility of its cellulose fraction, which is associated with a lower pretreatment efficiency on this biomass.

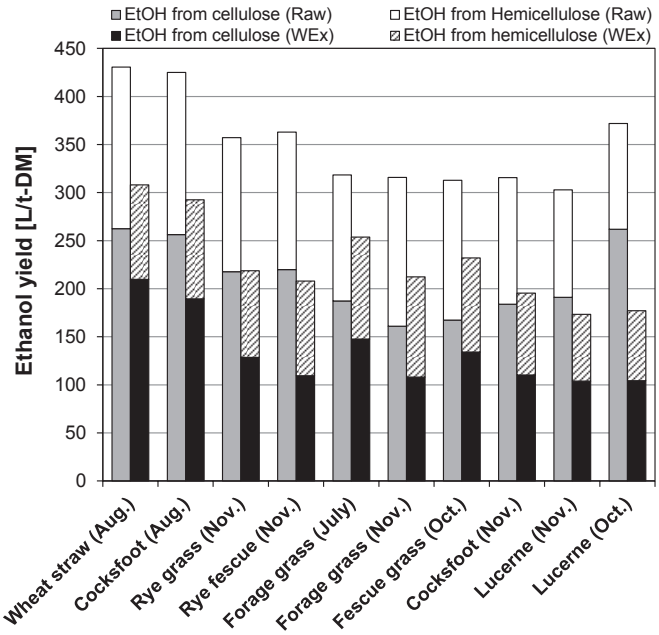

Fig. 4. Potential ethanol yield based on the composition of the raw biomass (left column for each biomass) and achievable ethanol yield based on C6 and C5 sugar yields after WEx pretreatment and enzymatic hydrolysis (right column for each biomass).

4. CONCLUSIONS

Among the biomasses studied, wheat straw and cocksfoot grass exhibited the highest bioethanol potential. The ethanol yield depended on biomass composition and pretreatment efficiency. Cellulose content of biomass harvested later in the year was generally lower. Hemicellulose and lignin contents and time of harvest were not correlated. Wet explosion with relatively low severity and dilute acid addition was effective for cocksfoot grass harvested in August, fescue grass, wheat straw, and forage grass. For the other biomass

resources, the severity of the pretreatment was too low, making further adjustment of the pretreatment parameters advisable.

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REFERENCES

Ahring, B. K., Westermann, P., 2007. Coproduction of Bioethanol with Other Biofuels. Advances in Biochemical Engineering Biotechnology 108, 289-302

American Public Health Association (APHA), American Water Works Association (AWWA), and Water Environment Federation (WAE) 1992. Standard Methods for the Examination of Water and Waste Water. (Ed. Greenberg, A. E., Clesceri, L. S., Trussel, R. R.) 18th edition.

Galbe, M., Zacchi, G., 2002. A review of the production of ethanol from softwood. Appl. Microbiol. Biotechnol. 59, 618-628.

Georgieva, T. I., Hou, X., Hilstrøm, T., Ahring, B. K., 2008. Enzymatic hydrolysis and ethanol fermentation of high dry matter wet-exploded wheat straw at low enzyme loading. Appl. Biochem. Biotechnol. 148, 35-44.

Hatzis, C., Riley, C., Philippidis, G. P., 1996. Detailed material balance and ethanol yield calculations for biomass-to-ethanol conversion process. Appl. Biochem. Biotechnol. 96, 0273-2289.

Hendriks, A. T. W. M., Zeeman, G., 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresource Technology 100, 10-18.

Himmel, M. E., Adney, W. S., Baker, J. O., Elander, R., McMillan, J. D., Nieves, R. A., Sheehan, J. J., Thomas, S. R., Vinzant, T. B., and Zhang, M., 1997. Advanced bioethanol production technologies: a perspective. ACS Symp. Ser. 666, 2-45.

Karimi, K., Emtiazi, G., Taherzadeh, M. J., 2006. Ethanol production from dilute–acid pretreated rice straw by simultaneous saccharification and fermentation with *Mucor indicus, Rhizopus oryzae, and Saccharomyces cerevisiae.* Enzyme and Microbial Technol. 40, 138-144.

Klinke, H. B., Thomsen, A. B., Ahring, B. K., 2004. Inhibition of ethanol–producing yeast and bacteria by degradation products produced during pre–treatment of biomass. Appl. Microbiol Biotechnol. 66, 10-26.

Knauf, M., Moniruzzaman, M., 2004. Lignocellulosic biomass processing: a perspective. Int. Sugar J. 106, 147-150.

Lu, X., Zhang, Y., Angelidaki, I., 2009. Optimization of H_2SO_4-catalyzed hydrothermal pretreatment of rapeseed straw for bioconversion to ethanol: Focusing on pretreatment at high solids content. Bioresource Technology 100, 3048-3053.

Lynd, L. R., 1996. Overview and evaluation of fuel ethanol from cellulosic biomass: Technology, Economics, the Environment, and Policy. Annual Review of Energy and Environment 21, 404-465.

Martin, C., Klinke, H. B., Thomsen, A. B., 2007. Wet oxidation as a pretreatment method for enhancing the enzymatic convertibility of sugarcane bagasse. Enzyme and Microbial Technology 40, 426-432.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M., Ladisch, M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technology 96, 673-686.

Palmqvist, E., Hahn-Hägerdal, B., 2000. Fermentation of lignocellulosic hydrolyzates II: inhibitors and mechanisms of inhibition. Bioresource Technology 74, 25-33.

Percival Zhang, Y. H., Berson, E., Sarkanen, S., Dale, B.E. 2009. Pretreatment and biomass recalcitrance: Fundamentals and Progress. Appl. Biochem. Biotechnol. 153, 80-83.

Petersen, M., Larsen, J., Thomsen, M. H., 2009. Optimization of hydrothermal pretreatment of wheat straw for production of bioethanol at low water consumption without addition of chemicals. Biomass and Bioenergy 33, 834-840.

Saha, B. C., 2004. Lignocellulose biodegradation and applications in biotechnology. ACS Symp. Ser. 889, 2-34.

Sassner, P., Mårtensson, C. G., Galbe, M., Zacchi, G., 2008. Steam pretreatment of H_2SO_4–impregnated Salix for the production of bioethanol. Bioresource Technology 99, 137-145.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., and Templeton, D., 2008a. Determination of Sugars, Byproducts and Degradation Products in Liquid Fraction Process Samples. Laboratory Analytical Procedure, Technical Report NREL/TP-510-42623.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., and Crocker, D., 2008b. Determination of Structural Carbohydrates and Lignin in Biomass. Laboratory Analytical Procedure, Technical Report NREL/TP-510-42618.

Sørensen, A., Teller, P. J., Hilstrøm, T., Ahring, B. K., 2008. Hydrolysis of miscanthus for bioethanol production using dilute acid presoaking combined with wet explosion pre-treatment and enzymatic treatment. Bioresource Technology 99, 6602-6607.

Thomsen, M. H., Thygesen, A., Jørgensen, H., Larsen, J., Christensen, B. H., and Thomsen, A. B., 2006. Preliminary results on optimization of pilot scale pretreatment of wheat straw used in coproduction of bioethanol and electricity. Appl. Biochem. and Biotechnol. 126-132, 448-460.

Varga, E., Schmidt, A. S., Reczey, K., Thomsen, A. B., 2003. Pretreatment of corn stover using Wet Oxidation to enhance enzymatic digestibility. Appl. Biochem. and Biotechnol. 104, 0273-2289.

Westermann, P., Ahring, B. K., 2005. The biorefinery for production of multiple biofuels. In: Lens, P., Westermann, P., Haberbauer, M., Moreno, A., editors. Biofuels for fuel cells: Renewable energy from biomass fermentation. IWA Publishing, 194–205.

Yang, E., Sharma-Shivappa, R. R., Burns, J. C., Cheng, J., 2009. Saccharification and fermentation of dilute acid pretreatment freeze-dried switchgrass. Energy Fuels 23, 5626-5635.

Research paper II

Tailoring wet explosion process parameters for the pretreatment of Cocksfoot grass for high sugar production

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In press in Applied Biochemistry and Biotechnology

Tailoring wet explosion process parameters for the pretreatment of Cocksfoot grass for high sugar production

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Abstract

The pretreatment of lignocellulosic biomass is crucial for efficient subsequent enzymatic hydrolysis and ethanol fermentation. In this study, wet explosion (WEx) pretreatment was applied to cocksfoot grass, based on WEx pretreatment of wheat straw as reference, and pretreatment conditions were tailored for maximizing the sugar yields after WEx pretreatment and enzymatic hydrolysis using response methodology (RSM). The WEx process parameters studied were temperature (160-210 ^{o}C), retention time (5-20 min), and dilute sulfuric acid concentration (0.2-0.5%). The pretreatment parameter set (E) 210 ^{o}C, 5 min, and 0.5% dilute sulfuric acid was found most suitable for achieving a high glucose release with low formation of byproducts. Under these conditions, a cellulose and hemicellulose sugar recovery was 94 and 70%, respectively. The efficiency of the enzymatic hydrolysis of cellulose under these conditions was 91% resulting in a calculated ethanol yield of approximately 330 L/ton DM based on total sugar yields from hexoses and pentoses. On the other hand, the released of pentose sugars was more pronounced under less severe pretreatment condition C (160 ^{o}C, 5 min, 0.2% dilute sulfuric acid). Therefore, pretreatment conditions should be tailored depending on the choice of products.

Keywords: Wet explosion; Lignocellulosic biomass; Cocksfoot grass; Pretreatment; Bioethanol yield; Response Surface Analysis.

1. INTRODUCTION

The utilization of agricultural residues, forestry, and organic waste materials for cellulosic biofuels production is a challenge for economical bioenergy production. These biomass resources are characterized by their lignocellulosic structure, that is highly complex, mainly composed of cellulose, hemicellulose, and lignin that are not directly accessible for microbial degradation (Chen and Qiu, 2010).

Lignin is a complex polymer of phenylpropane units and methoxy groups, linked in a three-dimensional structure which is particularly difficult to biodegrade. Lignin is the most recalcitrant component of the plant cell wall and protects the plant from physical and microbial degradation (Hendriks and Zeeman, 2009). The higher the proportion of lignin in the lignocellulosic biomass, the higher is its resistance to chemical and enzymatic degradation. It restricts hydrolysis by shielding cellulose surfaces and inactivating enzymes (Taherzadeh and Karimi, 2008). Cellulose, on the other hand, is extremely crystalline, water insoluble, and highly resistant to depolymerization, which makes it rather difficult for hydrolyzing enzymes to access (Sørensen et al., 2008). Therefore, release of glucose from cellulose faces significant technical challenges. Hemicelluloses are heterogeneous polymers of pentoses (xylose and arabinose) as the dominant sugars and small amounts of non-sugars such as acetyl groups. In contrast to cellulose, which is crystalline and strong, hemicelluloses have a random, amorphous, branched structure with little resistance to hydrolysis, and are relatively easy to hydrolyze to their sugar monomers by dilute acids (Hamelinck et al., 2005).

The biochemical conversion of lignocellulose into valuable products largely depends on an effective pretreatment technology (Himmel et al., 1997). Under the pretreatment, lignocellulosic biomass is converted from its native form, in which it is recalcitrant to cellulose enzyme systems, into a form for which cellulose hydrolysis is effective (Hendriks and Zeeman, 2009). The main purpose of the pretreatment is to break the lignocellulosic structure (Kumar et al., 2009), and to reduce the cellulose crystallinity with low loss of sugar compounds (Karimi et al., 2006). At the same time, the formation of degradation

products that inhibit the microbial activities during ethanol fermentation should be kept low (Mosier et al., 2005).

Lignin, on the other hand, is a valuable by-product which can be burned to provide heat and electricity, or utilized as raw material for the conversion into various polymeric compounds (Saha and Bothast, 1997). For improving the economy of the ethanol production process it is therefore, essential to recover the lignin fraction after pretreatment.

The current study is part of an optimization of the so-called BornBioFuel concept for cellulosic bioethanol production that is going to be implemented in a demonstration plant on the island of Bornholm, Denmark (Bacovsky et al., 2010). In the BornBioFuel concept, the previously developed wet explosion (WEx) pretreatment method (Westermann et al., 2005; Ahring and Westermann, 2007; Georgieva et al., 2008; Sørensen et al., 2008) was modified by addition of dilute sulfuric acid.

In a previous screening of different biomass resources available on the island of Bornholm, cocksfoot grass revealed a high potential as feedstock for the BornBioFuel demonstration plant (Njoku et al., 2012). Cocksfoot grass is an abundant biomass, widely distributed in Europe and United States. It is a hardy deep-rooted perennial grass, well suited to dry conditions and acid soils. Starting its growth in early spring, it can reach heights up to 140 cm; with the leaves reaching the lengths of 50 cm (Moore et al., 2006; Xu et al., 2006).

The overall objective of this study was to evaluate and increase the efficiency of the modified WEx pretreatment method with dilute sulfuric acid addition. The evaluation of the WEx pretreatment of cocksfoot grass was performed by adjusting the WEx process parameters for maximizing the sugar yields after WEx pretreatment and enzymatic hydrolysis.

2. MATERIAL AND METHODS

2.1. Raw material

Cocksfoot grass and wheat straw from the island of Bornholm, Denmark, were harvested in August. The biomass samples were air-dried and hammer milled to a particle size of 2-3

mm and stored in plastic bags at room temperature prior to pretreatment. A portion of the raw biomass was ground in a coffee grinder to a particle size of 1 mm and used for chemical composition analysis.

Dry matter content (DM), volatile solid content (VS), and ash were determined according to the procedure described by the American Public Health Association (APHA, 1992). The content of total carbohydrates (cellulose and hemicellulose), and Klason lignin in the raw biomass was determined by strong acid hydrolysis according to the procedure developed by the National Renewable Energy Laboratory (Sluiter et al., 2008a).

Sugar analysis (glucose and xylose) was performed by high performance liquid chromatography (HPLC) with refractive index (RI) detection (Shimadzu Corp., Japan) on an Aminex HPX-87H column (Bio-Rad Laboratories, CA, USA) using 4 mM H_2SO_4 as eluent and a flow rate of 0.6 mL/min at 60 ^oC. Prior to HPLC analysis, samples were centrifuged at 4000*g* for 10 min, and filtered through a 0.45 μm syringe filter.

The VS content of the raw biomass found as difference between the total VS value and the sum of the carbohydrate fractions analyzed in the raw biomass was referred to as "other organic matter", which is the sum of proteins, fats and volatile compounds.

2.2. Wet explosion pretreatment

The wet explosion pretreatment was performed in batches in a 5 L WEx reactor (Njoku et al., 2012), equipped with a high-pressure cylinder, a gas/liquid inlet for injection of dilute sulfuric acid, and a continuous stirrer (990 rpm). The reactor was heated by a water jacket connected to a heat exchanger controlled by an oil heater. The temperature inside the reactor was monitored by a temperature sensor mounted in the headspace. The pretreatment was performed by suspending 300 g raw biomass in 1600 g of tap water. The sulfuric acid was injected into the WEx reactor after reaching the desired temperature.

After the treatment, the biomass slurry was flashed into a 20 L flash tank connected to the reactor, resulting in an immediate drop in temperature and pressure.

The resulting biomass slurry from the pretreatment was separated into liquid and solid fractions. The separation was performed in a commercial filtration unit (Larox Buchner unit) with a filtering cloth pore size of 20 μm and a constant vacuum pressure of -0.7 psi.

The solid fraction was washed thoroughly with water and stored in a freezer (-18 ^{o}C) prior to compositional analysis and further processing. The separated liquid fraction was stored at 4 ^{o}C before further analyses.

2.3. Analysis of the solid fraction

A part of the solid fraction (washed filter cake) of the WEx slurry was dried in an incubator at 38 ^{o}C for 12 h before composition analysis. The dried solid fractions were ground in a coffee grinder to a 1 mm particle size before composition analysis. The content of total carbohydrates (cellulose and hemicellulose), and Klason lignin in the separated solid fractions, $Cell_{SF}$, $Hemicell_{SF}$, and $Lignin_{SF}$ was determined by strong acid hydrolysis as previously described (Sluiter., 2008a).

The recovery of cellulose and hemicelluloses was calculated by using Eq. (A.1) and (A.2):

$$\text{Cellulose (\%)} = \frac{\text{Cell}_{SF} + \text{Glucose}_{LF} / 1.11}{\text{Cell}_{RB}} \cdot 100 \qquad \text{(A.1)}$$

$$\text{Hemicellulose (\%)} = \frac{\text{Hemicell}_{SF} + (\text{Xylose}_{LF} + \text{Arabinose}_{LF}) / 1.14}{\text{Hemicell}_{RB}} \cdot 100 \qquad \text{(A.2)}$$

where $Glucose_{LF}$ is the glucose in the liquid fraction, $Cell_{SF}$ and $Cell_{RB}$ are the cellulose, and $Hemicell_{SF}$ and $Hemicell_{RB}$ is the hemicellulose found in the solid fraction, and in the raw biomass, respectively, while $Xylose_{LF}$ and $Arabinose_{LF}$ are the xylose and arabinose in the liquid fraction, respectively; and 1.11, and 1.14 are the stoichiometric conversion factors of polysaccharides to sugar monomers.

2.4. Enzymatic hydrolysis of solid fraction

The release of hydrolysable cellulose by the wet explosion pretreatment was analyzed by the sugars released after enzymatic hydrolysis using a commercial enzyme mixture (Cellic CTec2), kindly provided by Novozymes A/S (Bagsværd, Denmark). The enzymatic

conversion of the separated wet exploded solid fraction was carried out at 5% DM with 0.05 M succinate buffer (pH 5.0). The experiments were performed in duplicates in 2 mL Eppendorf tubes filled with 1.5 mL of hydrolysis media, and at an enzyme dosage of 20 mg-EP/g-VS (EP = enzyme protein) for all the samples. The hydrolysis mixture was incubated for 72 h at 50 ^{o}C in a thermomixer shaker at 1400 rpm. The reaction was stopped by heating the solution to 100 ^{o}C for 10 min, mixed by vortexing, and centrifuged for 8 min at 3600*g*. The concentration of glucose, xylose, and arabinose in the hydrolyzate was quantified by HPLC as described in Section 2.1.

The efficiency of cellulose hydrolysis was calculated according to (Eq. B.1): where Glucose $_{EH}$ is the mass of glucose released after enzymatic hydrolysis of cellulose in the solid fraction.

$$\text{Hydrolysis}_{\text{Cellulose}}(\%) = \frac{\text{Glucose}_{\text{EH}} / 1.11}{\text{Cell}_{\text{RB}}} \cdot 100 \qquad \text{(B.1)}$$

2.5. Analysis of the liquid fraction

Carbohydrates in the liquid fractions (filtrate) after WEx pretreatment were both polymers and oligomers together with small amounts of monomers, thus the samples were hydrolyzed using 4% (w/w) sulfuric acid at 121 ^{o}C for 10 min to determine the total xylose, arabinose, and glucose concentration in the filtrate. The analysis was determined according to the National Renewable Energy Laboratory protocol (Sluiter et al., 2008b). Glucose, xylose, arabinose, 2-furfural, hydroxymethylfurfural (HMF), and carboxylic acids (acetic acid and glycolic acid) were quantified by HPLC as described in Section 2.1. The yield of pentose sugars after WEx pretreatment was calculated according to (Eq. B.2): where Pentoses $_{LF}$ is the mass of the pentose sugars xylose and arabinose released after wet explosion pretreatment in the liquid fraction.

$$\text{Hydrolysis}_{\text{Hemicellulose}}(\%) = \frac{\text{Pentoses}_{\text{LF}} / 1.14}{\text{Hemicell}_{\text{RB}}} \cdot 100 \qquad \text{(B.2)}$$

The maximum achievable ethanol yield was calculated for the different pretreatment conditions based on full conversion of glucose and pentose sugars released after WEx pretreatment and enzymatic hydrolysis of the cellulose fraction according to (Eq. C):

$$\text{Yield}_{EtOH,tot} = \text{Glucose}_{tot} \times 0.51 + \text{Pentoses}_{tot} \times 0.50 \quad \text{(C)}$$

where Glucose_{tot} is the total glucose released after pretreatment and enzymatic hydrolysis and Pentoses_{tot} is the total pentoses released from hemicelluloses after WEx pretreatment, and 0.51 and 0.50 are the maximal achievable ethanol fermentation yields (in g-EtOH/g-sugar) from glucose and xylose, respectively, (Hatzis et al., 1996).

2.6. Response Surface Analysis

The adjustment of the WEx pretreatment parameters was based on response surface methodology (RSM); it was employed to reduce the total number of experiments needed to determine the most efficient combination of WEx process parameters for the pretreatment of cocksfoot grass. Three independent variables, temperature (X_1), retention time (X_2) and dilute acid concentration (X_3) were studied. Table 1 depicts the experimental design and the process parameters tested.

Table 1. Process conditions used for modified WEx pretreatment of cocksfoot grass.

Pretreatment condition #	Factor X_1	Factor X_2	Factor X_3
	Temperature [^{o}C]	Retention time [min]	Acid concentration [%]
A	210	20	0.5
B	160	20	0.35
C	160	5	0.2
D	210	5	0.2
E	210	5	0.5
F	160	20	0.5
G	210	20	0.35
H	185	13	0.2
I	160	11	0.5
J	180	5	0.35

The content of glucose Y_1 (% of theoretical yield), pentoses Y_2 (% of theoretical yield), furfural Y_3 (g/100 g DM), HMF Y_4 (g/100 g DM), and carboxylic acids Y_5 (g/100 g DM) after pretreatment of cocksfoot grass were chosen as the dependent variables or response of the experimental design. StatGraphics Plus (SGWIN Version 5.0) was used for regression and graphical analyses of the data obtained. The fitness of the polynomial model equation was expressed by the coefficient of determination R^2, and its statistical significance was checked by F-test at a probability value ($p < 0.01$ and < 0.05). The significances of the regression coefficient were also tested by F-test.

3. RESULTS AND DISCUSSION

3.1. Raw material composition

The chemical composition of cocksfoot grass and wheat straw were quite similar with a slightly lower content of cellulose and lignin, but a slightly higher content of hemicelluloses and other organic matter for cocksfoot grass (Table 2).

Table 2. Composition analysis of raw biomass.

	Samples of raw biomass	
	Wheat straw	Cocksfoot grass
g-DM/100 g biomass	92.7	92.9
	[a]Composition biomass [g/100g-DM]	
Cellulose	36.5 (0.09)	35.6 (0.31)
Hemicelluloses	23.3 (0.65)	23.4 (0.06)
Klason lignin	19.5 (0.03)	18.8 (0.04)
Ash	5.2 (0.02)	5.2 (0.04)
Other organic matter	15.5 (0.00)	17.0 (0.00)

[a]All values are averages of two replicates. Standard deviation shown in parentheses.

3.2. WEx pretreatment and recovery of carbohydrates and lignin

The wet explosion (WEx) pretreatment with dilute acid addition fractionated the lignocellulosic materials into a solid fraction containing mainly cellulose and lignin, and a liquid fraction enriched with solubilized hemicelluloses, mainly present as pentose sugars and low molecular lignin fragments in dissolved form.

The recovery of cellulose in the solid fraction after WEx pretreatment varied between 94% for pretreatment condition A (210 ^{o}C, 20 min, 0.5% acid) and 97% for condition C (160 ^{o}C, 5 min, 0.2% acid) (Fig. 1). The variation of the hemicellulose recovery was much higher, between 67% for condition A (210 ^{o}C, 20 min, 0.5% acid) and 96% % for condition C (160 ^{o}C, 5 min, 0.2% acid). The lower recoveries under the pretreatment with the highest severity (A, D, E, and G) indicate a significant sugar conversion to other products, such as furfural and HMF during the WEx process. This makes dilute acid WEx pretreatment at lower severity, specifically condition C (160 ^{o}C, 5 min, 0.2% acid), more suitable for achieving high recoveries of both cellulose and hemicellulose from cocksfoot grass. In comparison, Georgieva et al. (2008) achieved 93 and 72% recovery of cellulose and hemicellulose from wheat straw with wet explosion pretreatment at 185 ^{o}C for 15 min and 35% (*v/v*) hydrogen peroxide. Martin et al. (2007) achieved cellulose and hemicellulose recovery of 70 and 93% respectively, after treating sugarcane bagasse by wet oxidation at 195 ^{o}C, 15 min, at alkaline pH.

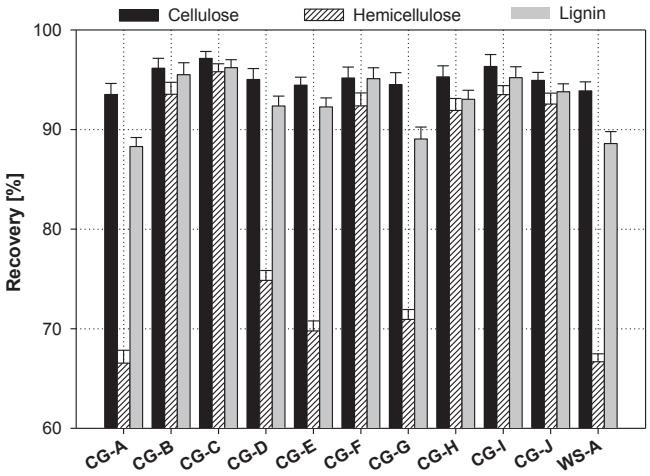

Fig. 1. Recovery of polysaccharides (cellulose and hemicellulose) and lignin after modified WEx pretreatment at different process conditions (error bars represent standard deviations of duplicates).

The lignin recovery in the solid fraction was more than 88% for all WEx pretreatment conditions (Fig. 1). Lignin recoveries above 95% were found for WEx conditions (B, C, F, and I), while recoveries below 90% were found for condition A (210 ^{o}C, 20 min, 0.5% acid) and G (210 ^{o}C, 20 min, 0.35% acid). This revealed that at more severe pretreatment conditions some of the lignin dissolved and degraded to low molecular lignin compounds. The highest degree of lignin solubilization was achieved after pretreatment at those conditions with high temperature and long retention time (A and G).

3.3. Enzymatic hydrolysis of cellulose in the solid fraction and yield of sugars in the liquid fraction after pretreatment

The enzymatic convertibility of cellulose under the different WEx conditions is one of the most important factors to evaluate the efficiency of the pretreatment for the production of bioethanol from C6 sugars (Varga et al., 2003) as it reveals the efficiency of enzymatic hydrolysis specifically for a certain enzyme mixture on a specific hydrolyzate.

Fig. 2A depicts the yields of glucose and pentoses after enzymatic hydrolysis of the wet-exploded solid fraction (filter cake). WEx pretreatment at 160 ^{o}C was not effective for improving the enzymatic hydrolysis of the cellulose fraction, as evident by low enzymatic convertibility of the cellulose released under the pretreatment conditions (B, C, F, and I). For the most severe tested pretreatment condition A (210 ^{o}C, 20 min, 0.5% acid), enzymatic convertibility of cellulose was 92 and 94% for cocksfoot grass and wheat straw, respectively. This is significantly higher than what Sørensen et al. (2008) reported for wet explosion pretreatment of miscanthus at 170 ^{o}C, 5 min, and 35% hydrogen peroxide achieving 58.5% glucose yield after enzymatic hydrolysis of the pre-soaked and wet exploded samples. Thomsen et al. (2006) reported 67-68% glucose yield from the solid fraction after hydrothermal pretreatment of wheat straw at 190 ^{o}C to 200 ^{o}C.

It has been previously demonstrated that a high pretreatment temperature and prolonged retention time could maximize glucose yields from cellulose materials (Taherzadeh and Niklasson, 2004); while it is more advantageous to apply a lower pretreatment temperature with regard to higher xylose yields from the hemicellulose fraction (Sørensen et al., 2008). The increase of the enzymatic convertibility of cellulose under pretreatment with high

severity is mostly linked to the high solubilization of hemicelluloses and removal of lignin fractions during pretreatment (Lee et al., 2008; Bjerre and Schmidt, 1997; Brodeur et al., 2011).

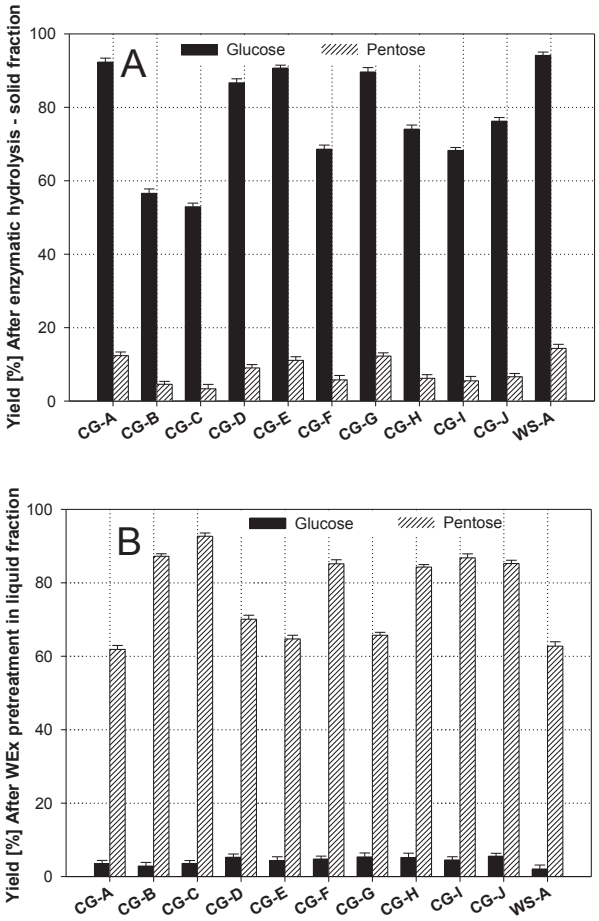

Fig. 2(A). Yield of glucose and pentoses after enzymatic hydrolysis of solid fractions. **(B).** Yield of glucose and pentoses in liquid fraction after modified WEx pretreatment. Average values and standard deviation reported for duplicates.

The lowest glucose yield was obtained at WEx pretreatment condition C, although this condition achieved the highest cellulose and hemicellulose recovery. These findings are in agreement with the proposition that the optimum conditions for the highest sugar recovery do not necessary mean the most effective conditions for enzymatic conversion of cellulose fractions to sugars monomers (Taherzadeh and Karimi, 2008).

The yields of solubilized pentoses (xylose, and arabinose), and glucose in the liquid fractions after WEx pretreatment are shown in Fig. 2B. Pentose yields above 85% can be found with pretreatment conditions B, C, F, H, I, and J, with the highest of 93% for condition C (160 ^{o}C, 5 min, and 0.2% acid). In comparison, Lu et al. (2009) found a xylose yield of 70% for 0.5-2% sulfuric acid-catalyzed hydrothermal pretreatment (180 ^{o}C, 5-20 min) of rapeseed straw. The most severe pretreatment conditions (A, D, E, and G) showed high degrees of hemicellulose solubilization, and about 30% of the solubilized hemicellulose sugars were degraded during the pretreatment, hence, resulted in the lowest yields of pentose sugars ranging from 62 to 70%.

3.4. Formation of degradation products during WEx pretreatment

Table 3 presents the concentration of carboxylic acids, furfural, and HMF in the liquid fraction after WEx pretreatment. Carboxylic acids were analyzed as the sum of acetic and glycolic acid. Depending on process severity, carboxylic acids, furan derivatives (furfural and 5-hydroxymethyl furfural-HMF), and phenolic compounds were generated during pretreatment of lignocellulosic biomass. These products are considered potential fermentation inhibitors (Saha, 2004; Klinke et al., 2004; Mosier et al., 2005). At more severe pretreatment conditions, xylose is degraded to furfural while HMF is formed from hexose degradation, and phenolic compounds are generated from partial breakdown of lignin. However, up to a certain limit, microorganisms can survive the stress of these compounds, but cell death would occur if the stress exceeds the limit that the cell can bear (Palmqvist and Hahn-Hägerdal, 2000). Accordingly, the highest by-products formation was found with the most severe pretreatment condition (A) at levels of 3.3 g/100 g DM for carboxylic acids, 2.3 g/100 g DM for furfural, and 0.48 g/100 g DM for HMF, respectively. These concentrations were higher after pretreatment of cocksfoot grass than of wheat straw.

For all pretreatment conditions of cocksfoot grass the concentration of carboxylic acids was generally higher than of the two by-products (furfural and HMF).

Table 3. Formation of by-products (g/100 g DM) in liquid fraction after modified WEx pretreatment.

Conditions	Carboxylic acids[a] g/100g DM	Furfural g/100g DM	HMF g/100g DM
CG-A	3.29 (0.06)	2.31 (0.03)	0.48 (0.01)
CG-B	1.62 (0.03)	0.05 (0.00)	0.03 (0.02)
CG-C	1.60 (0.04)	0.04 (0.01)	0.02 (0.00)
CG-D	2.69 (0.07)	0.61 (0.04)	0.09 (0.01)
CG-E	2.96 (0.03)	1.04 (0.01)	0.10 (0.00)
CG-F	1.86 (0.06)	0.09 (0.03)	0.05 (0.02)
CG-G	2.13 (0.00)	1.13 (0.01)	0.59 (0.00)
CG-H	1.97 (0.05)	0.18 (0.04)	0.04 (0.01)
CG-I	1.67 (0.08)	0.07 (0.02)	0.05 (0.04)
CG-J	1.85 (0.00)	0.10 (0.03)	0.06 (0.00)
WS-A	2.90 (0.02)	1.76 (0.08)	0.80 (0.00)

Average of duplicates. Standard deviation shown in parentheses. [a]Sum of acetic acid and glycolic acid.

High concentration of carboxylic acids was also pronounced under pretreatment conditions (D, E, and G), with more than 2 g/100 g DM. On the other hand, the concentrations of the by-products were significantly lower for conditions with lower pretreatment severity (B, C, F, and I).

For all pretreatments at 160 ^{o}C (B, C, F, and I), the concentration was 0.1 g/100 g DM and lower for furfural and 0.05 g/100 g DM and lower for HMF. In comparison, Martin et al. (2007) found 9.21 g/100 g material of carboxylic acids, 0.53 g /100 g material for furfural, and 0.07 g/100 g material for HMF after wet oxidation pretreatment of sugarcane bagasse at 195 ^{o}C, 15 min, at alkaline pH.

3.5. Theoretical ethanol production and overall mass balance for WEx process

The maximum achievable ethanol yields for the different pretreatment conditions are presented in Fig. 3. The total ethanol yield from both glucose and pentose sugars after WEx pretreatment and enzymatic hydrolysis ranged for cocksfoot grass from 275 L/ton DM at condition B to 330 L/ton DM at condition A, compared to 341 L/ton DM for wheat straw at condition A. For the highest total yield, the ethanol yield from glucose was corresponding to 92% and 94% of the theoretical maximum for cocksfoot grass and wheat straw, respectively, while the ethanol yields from pentose sugars was 56% of the potential.

For pretreatment with lower severity the contribution of ethanol from the pentose sugars in the liquid fraction does not increase as much as the ethanol yield from glucose after enzymatic hydrolysis of the solid fraction decreases, resulting in a lower total ethanol yield, with the lowest values of 276 L/ton DM and 275 L/ton DM for pretreatment conditions B and C.

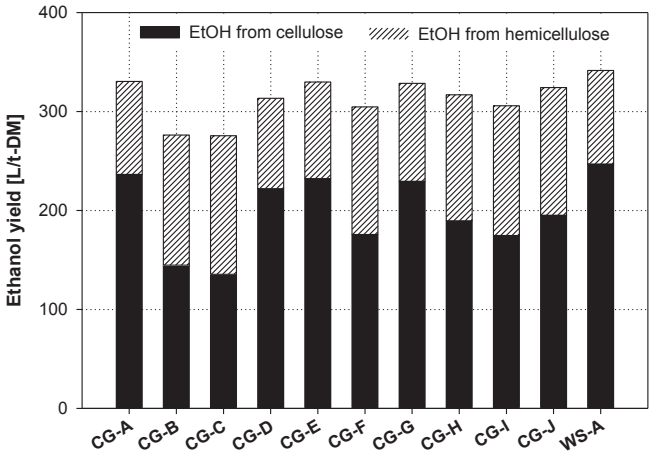

Fig. 3. Achievable ethanol yield based on the yield of glucose and pentose sugar yields after modified WEx pretreatment and enzymatic hydrolysis at different conditions.

Mass balance calculations are necessary for evaluating the efficiency of a pretreatment method applied for fractionation of lignocellulosic biomass (Hatzis et al., 1996). Hence, the

overall sugar recoveries and yields can then be quickly gained from the mass balance calculations over the different conversion process steps. The WEx pretreatment performed at 210 ^{o}C with 0.5% acid concentration for 5 min, was the best pretreatment parameters for maximizing the glucose production due to its low production of degradation products and relatively high pentose sugars released in the liquid fraction compared with relatively lower yield with pretreatment condition at 210 ^{o}C for 20 min with 0.5% acid concentration. For this pretreatment condition, 336.4 g cellulose was recovered from 356.2 g cellulose input to the WEx pretreatment process; also, about 135.6 g xylose and 15.2 g glucose were released in the liquid fraction during the WEx process (Fig. 4).

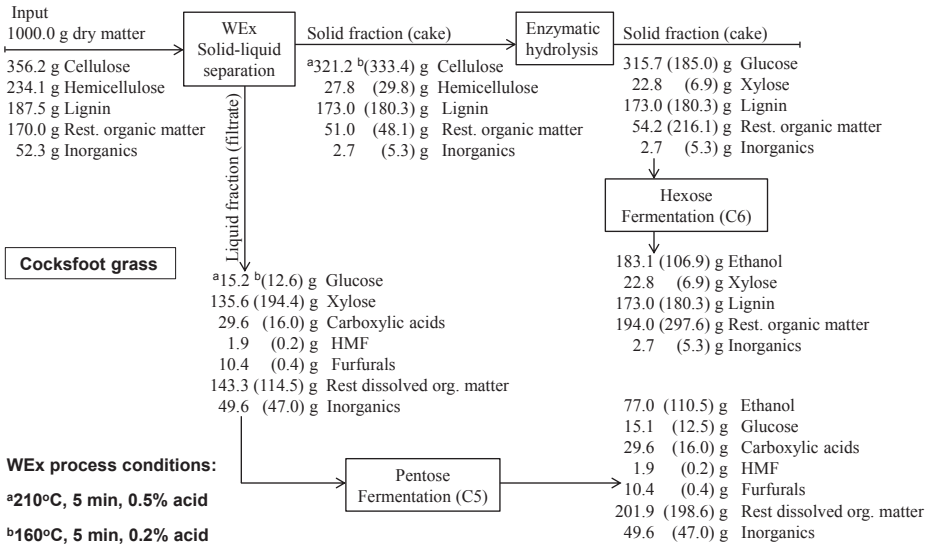

Fig. 4. Mass balance for the pretreatment, hydrolysis, and achievable ethanol yield for modified WEx pretreatment.

Subsequent enzymatic hydrolysis of the solid fraction after the WEx pretreatment, released 315.7 g glucose and 22.8 g xylose. The mass balance calculations shows that approximately 58% of the hemicellulose was converted to monomeric xylose by the WEx

pretreatment and about 90% of the cellulose was hydrolyzed to monomeric glucose. Following the sugars released from the pretreatment and subsequent enzymatic hydrolysis, 183.2 g ethanol from the released glucose could be potentially achieved and 77.0 g ethanol from the released pentose sugars can be potentially gained if fermented to ethanol by ethanologenic strains (*Saccharomyces cerevisiae* or *Pichia stipitis*). However, the highest solubilized hemicellulose sugars were achieved with pretreatment condition at 160 ^{o}C with 0.2% acid concentration for 5 min. A total of 194.4 g hemicellulose sugars were recovered from 234.1 g hemicellulose input to the WEx pretreatment process for this condition (see Fig. 4), the values in brackets. It is obvious that the release of hexose and pentose sugars needs different pretreatment severity, as the conditions where the best hexose sugars were obtained is detrimental to the release of pentose sugars. It is therefore, important to find a meeting point in the pretreatment parameters where both hexose and pentose sugars can be achieved at higher yields depending on the product choice of interest.

3.6. Influence of pretreatment parameters combinations

The significant of the different WEx pretreatment parameters and their interaction effects was determined by analysis of variance (ANOVA), which tests the statistical significance of each effect by comparing the mean square against an estimate of the experimental error. The results of ANOVA confirmed the effects suggested by direct data analysis. A regression analysis was performed to attain a mathematical model that better describes the relation between independent variables and the studied responses (glucose, pentose, furfural, HMF, carboxylic acids). ANOVA for glucose yield after WEx pretreatment and enzymatic hydrolysis is presented in Table 4. The ANOVA of the regression model proves that the model is highly significant as is evident by the calculated *F*-value (61.61) and a very low p-value ($p < 0.0002$). The high value obtained in *F*-value indicates that most of the variation in glucose yield can be explained by the regression model equation. The model presented a good determination coefficient ($R^2 = 0.98$). The determination coefficient model for other response variables are: ($R^2 = 0.93$) for Y_2, ($R^2 = 0.94$) for Y_3, ($R^2 = 0.90$) for Y_4, and ($R^2 = 0.95$) for Y_5, showing a close agreement between experimental and the theoretical values predicted by the first-order polynomial results. The

statistical analysis demonstrate that the variation of the glucose yield was strongly dependent on pretreatment temperature (X_1) with a high F-value (196.7) and less dependent on retention time (X_2) and acid concentration (X_3) (see Table 4).

Table 4. Analysis of variance (ANOVA) table for the significant effects on glucose yield.

Sources of variation	Sum of squares	Degrees of freedom	Mean squares	F-Value	P-Value
Model	1339.68	4	331.67	61.61	< 0.0002
X_1	1018.76	1	1018.76	196.66**	< 0.0000
X_2	180.92	1	180.92	41.04*	< 0.0014
X_3	45.51	1	45.51	8.45	< 0.0335
X_1X_2	94.49	1	94.49	14.28	< 0.0218
Error	13.92	5	5.38		
Total	1353.60	9			

The first-order polynomial equations representing the model in terms of actual units for the different response variables are depicted in Eq. (D.1, D.2, D.3, D.4, and D.5):

$$Y_1\,(\%) = -36.1021 + 0.504332X_1 + 0.805353X_2 + 31.1867\,X_3 - 0.00248139X_1X_2 \quad \text{(D.1)}$$

$$Y_2\,(\%) = 118.15 - 0.210873X_1 - 0.0517493X_2 + 29.761X_3 - 0.195634X_1X_3 \quad \text{(D.2)}$$

$$Y_3\,(\text{g/100g DM}) = 0.775175 - 0.0059607X_1 - 0.162983X_2 - 8.56962X_3 + 0.00109896X_1X_2 + 0.0531185X_1X_3 \quad \text{(D.3)}$$

$$Y_4\,(\text{g/100g DM}) = 0.0282526 - 0.00017042X_1 - 0.0845465X_2 + 0.000526598X_1X_2 \quad \text{(D.4)}$$

$$Y_5\,(\text{g/100g DM}) = 1.96965 + 0.00523907X_1 - 0.0798705X_2 - 12.238X_3 + 0.0573047X_1X_3 + 0.211431X_2X_3 \quad \text{(D.5)}$$

The three-dimensional response surface plot described by the above-mentioned first-order polynomial equation was fitted to the experimental data points for glucose yields as

represented in Fig. 5A. The shapes of response surfaces indicate the nature and extent of the interaction between different independent variables (Prakash et al., 2008). The surface plots for the glucose yields in Fig. 5 A1 and A2 show that the pretreatment temperature had the major increasing effect on the glucose yield. As shown in Fig. 5A1, higher acid concentration lead to a higher glucose yield only for the pretreatment at lower temperatures, while the effect of higher acid addition was negligible for higher treatment temperatures. At lower treatment temperatures prolonging the retention time up to 12 minutes increased the glucose yield while a longer retention time had a negative effect (Fig. 5 A2). At temperatures above 180 ^{o}C the effect of prolonging the retention time to more than 5 minutes on the glucose yield was negligible. Overall, the glucose yield was highest at 210 ^{o}C with only a small further increase with increasing the acid concentration to more than 0.2% and the retention time to more than 5 min.

Regarding the response surface plots for the pentose yield (Fig. 5 B1 and B2), the response surface showed that temperature has again the major influence, but now the yield is decreasing with higher temperatures. Increasing the acid concentration does have no or rather a negative effect on the pentose yield at higher temperatures. Also the increase of the retention time has a negative effect on the pentose yield. Accordingly, the best pretreatment conditions for achieving the highest pentose yield are 160 ^{o}C, 0.2%, and 5 min retention time. The effect of temperature, acid concentration and retention time on the furfural and HMF production is shown in Fig. 6 A1, A2 and Fig. 6 B1, B2, respectively. Furfural and HMF production was significantly increasing at temperatures above 180^{o}C (Fig. 6 A1, A2, B1, and B2). An increasing acid concentration only led to higher furfural concentrations at temperatures above 180 ^{o}C. Prolonging the retention time had no significant effect at 160 ^{o}C, but was leading to higher furfural and HMF concentrations at higher pretreatment temperatures (Fig. 6 A2 and B2). Overall, pretreatment at 180 ^{o}C and 5 min retention time was leading to low furfural and HMF production, independent of the applied acid concentration. The production of carboxylic acids was mainly affected by an increase in treatment temperature, while acid concentration and retention time had only a significant effect for pretreatment at temperatures higher than 180 ^{o}C (Fig. 6 C1 and C2).

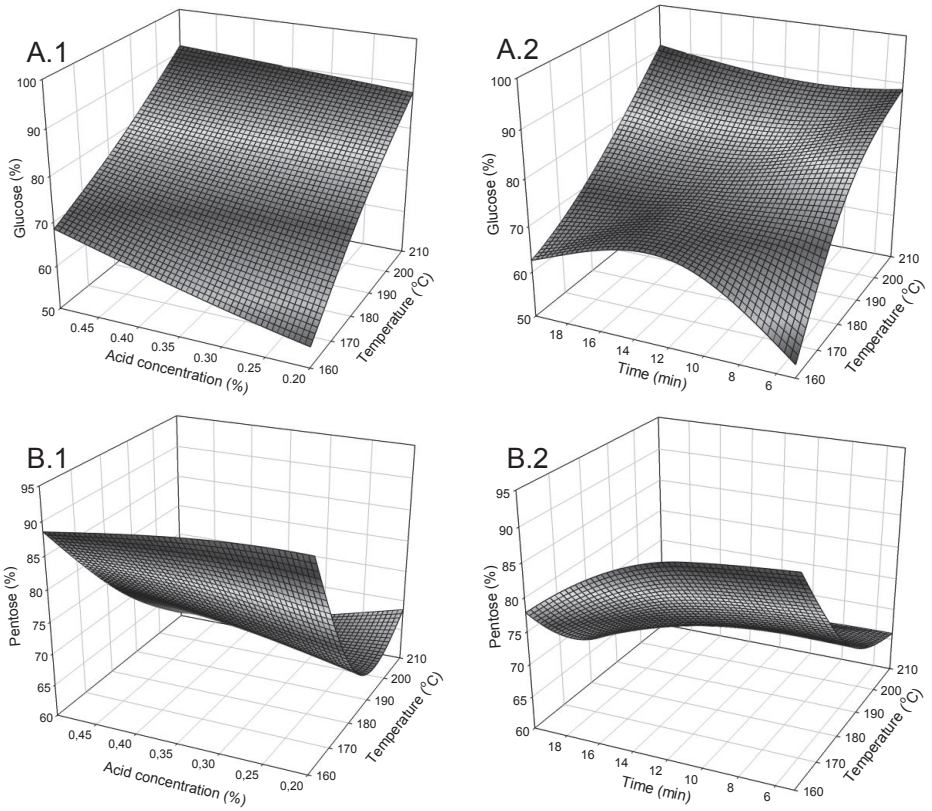

Fig. 5(A). Response surface plot on glucose yield in the solid fraction. A1 – Effect of acid concentration and treatment temperature at retention time of 20 min. A2 – Effect of retention time and treatment temperature at acid concentration of 0.5%. **(B).** Response surface plot on pentose yield in the liquid fraction. B1 – Effect of acid concentration and treatment temperature at retention time of 5 min. B2 – Effect of retention time and treatment temperature at acid concentration of 0.2%.

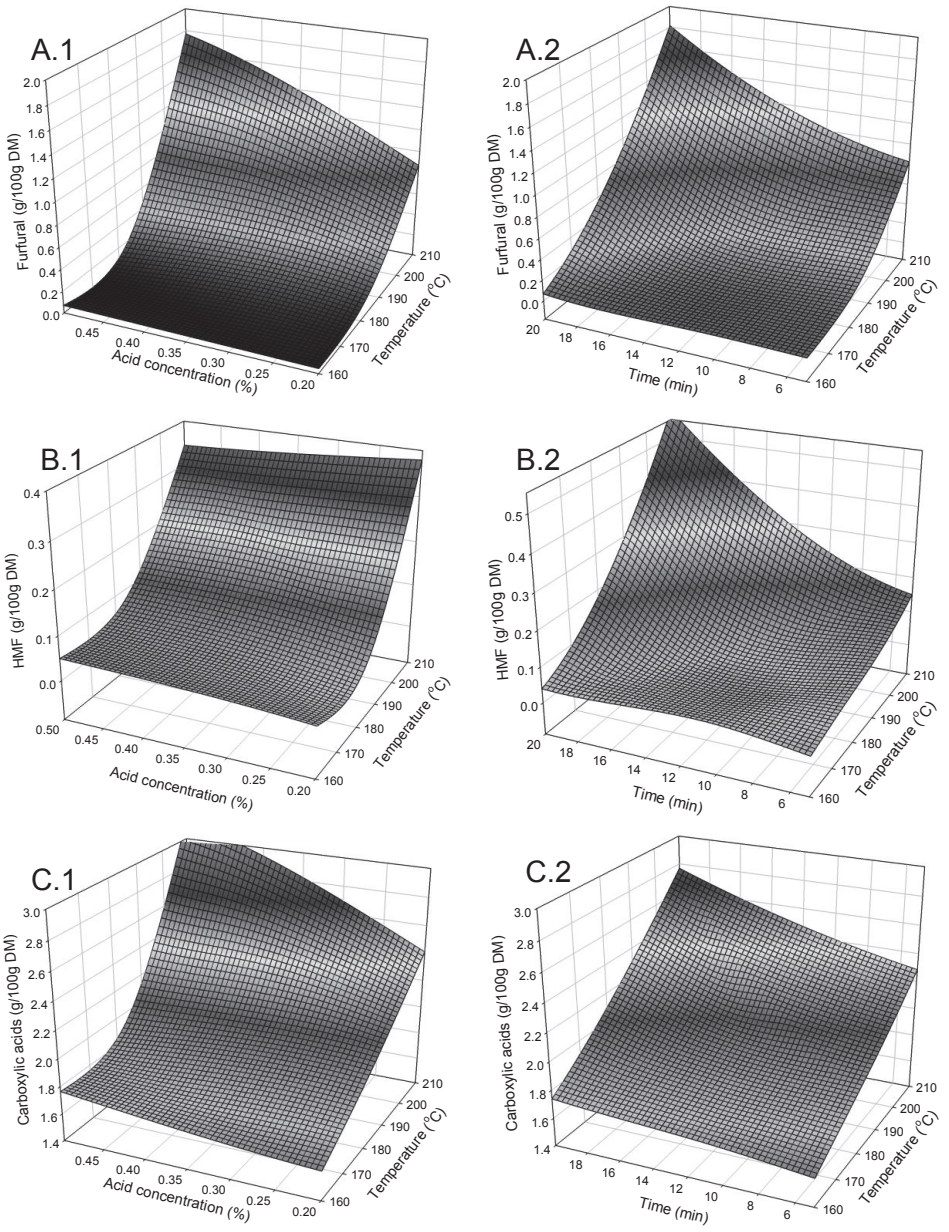

Fig. 6(A). Response surface plot on furfural production. A1 – Effect of acid concentration and treatment temperature at retention time of 20 min. A2 – Effect of retention time and

treatment temperature at acid concentration of 0.5%. **(B).** Response surface plot on HMF formation. B1 – Effect of acid concentration and treatment temperature at retention time of 20 min. B2 – Effect of retention time and treatment temperature at acid concentration of 0.5%. **(C).** Response surface plot on carboxylic acids production. C1 – Effect of acid concentration and treatment temperature at retention time of 20 min. C2 – Effect of retention time and treatment temperature at acid concentration of 0.5%.

4. CONCLUSIONS

The most suitable conditions for the WEx pretreatment of cocksfoot grass with respect to achieving reasonable ethanol yield from both glucose and pentose sugars were 210 ^{o}C, 0.5% acid concentration and 5 min retention time. This condition resulted in approximately 91% glucose yield for cocksfoot grass. The highest pentose yield (93%) was found at lower pretreatment severity (160 ^{o}C, 0.2% acids, 5 min), while at most severe WEx conditions this value significantly decreased to around 60%. Apparently, the release of hexose and pentose sugars needs different pretreatment conditions, as the conditions where the best hexose sugars were obtained is unfavorable to the release of pentose sugars. It is therefore, crucial to find a middle pretreatment severity in order to maximize the release of fermentable sugars for production of high-value biobased products both for hexose and pentose sugars. From our findings, it is suggested that pretreatment process parameters should be tailored to the specific biomass compositional structures and with a view to all the potential sugars which can be produced enabling economic feasible process.

ACKNOWLEDGMENTS

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REFERENCES

Ahring, B. K., Westermann, P., 2007. Coproduction of bioethanol with other biofuels. Advances in Biochem. Engineering Biotechnol. 108, 289-302.

American Public Health Association (APHA), American Water Works Association (AWWA), and Water Environment Federation (WAE), 1992. Standard Methods for the Examination of Water and Waste Water. (Ed. Greenberg, A. E., Clesceri, L. S., Trussel, R. R.), 18th edition.

Bacovsky, D., Dallos, M., Wörgetter, M., 2010. Status of 2nd generation biofuels demonstration facilities. IEA Bioenergy Task 39 Report T39-P1b.

Bjerre, A. B., Schmidt, A. S., 1997. Development of chemical and biological processes for production of bioethanol: Optimization of the wet oxidation process and the characterization of products. Risø-R-967(EN), Rise National Laboratory, Denmark.

Brodeur, G., Yau, E., Badal, K., Collier, J., Ramachandran, K. B., Ramakrishnan, S., 2011. Chemical and physicochemical pretreatment of lignocellulosic biomass: A review. Enzyme Research, doi: 4061/2011/787532.

Chen, H., Qiu, W., 2010. Key technologies for bioethanol production from lignocellulose. Biotechnol. Advances 28, 556-562.

Georgieva, T. I., Hou, X., Hilstrøm, T., Ahring, B. K., 2008. Enzymatic hydrolysis and ethanol fermentation of high dry matter wet-exploded wheat straw at low enzyme loading. Appl. Biochem. Biotechnol. 148, 35-44.

Hamelinck, C. N., Hooijdonk, V. G., Faaij, A. P. C., 2005. Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle-and long-term. Biomass and Bioenergy 28, 384-410.

Hatzis, C., Riley, C., Philippidis, G. P., 1996. Detailed material balance and ethanol yield calculations for biomass-to-ethanol conversion process. Appl. Biochem. Biotechnol. 96, 0273-2289.

Hendriks, A. T. W. M., Zeeman, G., 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresource Technology 100, 10-18.

Himmel, M. E., Adney, W. S., Baker, J. O., Elander, R., McMillan, J. D., Nieves, R. A., Sheehan, J. J., Thomas, S. R., Vinzant, T. B., and Zhang, M., 1997. Advanced bioethanol production technologies: a perspective. ACS Symp. Ser. 666, 2-45

Karimi, K., Emtiazi, G., Taherzadeh, M. J., 2006. Ethanol production from dilute–acid pretreated rice straw by simultaneous saccharification and fermentation with *Mucor indicus, Rhizopus oryzae, and Saccharomyces cerevisiae.* Enzyme and Microbial Technol. 40, 138-144.

Klinke, H. B., Thomsen, A. B., Ahring, B. K., 2004. Inhibition of ethanol–producing yeast and bacteria by degradation products produced during pre–tretreatment of biomass. Appl. Microbiol Biotechnol. 66, 10-26.

Kumar, P., Barrett, D. M., Delwiche, M. J., and Stroeve, P., 2009. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind. Eng. Chem. Res. 48, 3713-3729.

Lee, J-S., Parameswaran, B., Lee, J-P., Park, S-C., 2008. Recent developments of key technologies on cellulosic ethanol production. Jour. Sci. Industrial Research 67, 865-873.

Lu, X., Zhang, Y., Angelidaki, I., 2009. Optimization of H_2SO_4-catalyzed hydrothermal pretreatment of rapeseed straw for bioconversion to ethanol: Focusing on pretreatment at high solids content. Bioresource Technology 100, 3048-3053.

Martin, C., Klinke, H. B., Thomsen, A. B., 2007. Wet oxidation as a pretreatment method for enhancing the enzymatic convertibility of sugarcane bagasse. Enzyme and Microbial Technology 40, 426-432.

Moore, G., Sanford, P., Wiley, T., 2006. Perennial pastures for Western Australia. Department of Agriculture and Food Western Australia, Bulletin 4690, Perth. <http://www.agric.wa.gov.au/objtwr/imported_assets/content/past/cocksfoot.pdf>, (Nov. 25, 2010).

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M., Ladisch, M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technology 96, 673-686.

Njoku, S. I., Ahring, B. K., Uellendahl, H., 2012. Pretreatment as the crucial step for a cellulosic ethanol biorefinery: Testing the efficiency of wet explosion on different types of biomass. Bioresource Technology 124, 105-110.

Palmqvist, E., Hahn-Hägerdal, B., 2000. Fermentation of lignocellulosic hydrolysates II: inhibitors and mechanisms of inhibition. Bioresource Technology 74, 25-33.

Prakash, O., Talat, M., Hasan, S. H., Pandey, R. K., 2008. Factorial design for the optimization of enzymatic detection of cadmium in aqueous solution using immobilized urease from vegetable waste. Bioresource Technology 99, 7565-7572.

Saha, B. C., 2004. Lignocellulose biodegradation and applications in biotechnology. ACS Symp. Ser. 889, 2-34.

Saha, B. C., Bothast, R. J., 1997. Enzymes in lignocellulosic biomass conversion. ACS Symp. Ser. 666, 46-56.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., and Crocker, D., 2008a. Determination of Structural Carbohydrates and Lignin in Biomass. Laboratory Analytical Procedure, Technical Report NREL/TP-510-42618.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., and Templeton, D., 2008b. Determination of Sugars, Byproducts and Degradation Products in Liquid Fraction Process Samples. Laboratory Analytical Procedure, Technical Report NREL/TP-510-42623.

Sørensen, A., Teller, P. J., Hilstrøm, T., Ahring, B. K., 2008. Hydrolysis of miscanthus for bioethanol production using dilute acid presoaking combined with wet explosion pre-treatment and enzymatic treatment. Bioresource Technology 99, 6602-6607.

Taherzadeh, M. J., Karimi, K., 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production. Int. J. Sci. 9, 1621-1651.

Taherzadeh, M. J., Niklasson, C., 2004. Ethanol from lignocellulosic materials: pretreatment, acid and enzymatic hydrolysis, and fermentation. ACS Symp. Ser. 889, 49-68.

Thomsen, M. H., Thygesen, A., Jørgensen, H., Larsen, J., Christensen, B. H., and Thomsen, A. B., 2006. Preliminary results on optimization of pilot scale pretreatment of wheat

straw used in coproduction of bioethanol and electricity. Appl. Biochem. And Biotechnol. 126-132, 448-460.

Varga, E., Schmidt, A. S., Reczey, K., Thomsen, A. B., 2003. Pretreatment of corn stover using Wet Oxidation to enhance enzymatic digestibility. Appl. Biochem. and Biotechnol. 104, 0273-2289.

Westermann, P., Ahring, B. K., 2005. The biorefinery for production of multiple biofuels. In: Lens, P., Westermann, P., Haberauer, M., Moreno, A., editors. Biofuels for fuel cells: Renewable energy from biomass fermentation. IWA Publishing, 194-205.

Xu, F., Geng, Z. C., Sun, J. X., Liu, C. F., Ren, J. L., Sun, R. C., Fowler, P., and Baird, M. S., 2006. Fractional and structural characterization of hemicelluloses from perennial ryegrass (*Lolium perenne*) and cocksfoot grass (*Dactylis glomerata*). Carbohydrate Research 341,2073-2082.

Research paper III

Comparing oxidative and dilute acid wet explosion pretreatment of Cocksfoot grass at high dry matter concentration for cellulosic ethanol production

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Comparing oxidative and dilute acid wet explosion pretreatment of Cocksfoot grass at high dry matter concentration for cellulosic ethanol production

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Abstract

The choice of a suitable pretreatment method and the adjustment of the pretreatment parameters for efficient conversion of biomass are crucial for a successful biorefinery concept. In this study, cocksfoot grass, a suitable lignocellulosic biomass with a potential for large scale production was investigated for cellulosic ethanol production. The biomass raw materials was pretreated using wet explosion (WEx) at 25% dry matter concentration with addition of oxygen or dilute sulfuric acid. The enzymatic hydrolysis of cellulose was significantly improved after pretreatment. The highest conversion into cellulose monomeric C6 sugars was achieved for WEx condition *AC-E* (180 ^{o}C, 15 min, and 0.2% sulfuric acid). For that condition, the highest ethanol yield of 250 mL/kg DM (97% of theoretical maximum value) was achieved for SSF process by *Saccharomyces cerevisiae*. However, the highest concentration of hemicellulose C5 sugars was found for WEx pretreatment condition *O2-A* (160 ^{o}C, 15 min, and 6 bar O_2) which means that the highest potential ethanol yield was found at these moderate pretreatment condition with oxygen added. Increasing the pretreatment temperature to 180-190 ^{o}C with addition of oxygen or dilute sulfuric acid significantly degrades the solubilized hemicellulose sugars and thus, achieved the highest formation of byproducts, such as acetic acid and furfural with a lower potential ethanol yield.

Keywords: Wet explosion; Lignocellulosic biomass; Cocksfoot grass; Pretreatment; Saccharification; Ethanol fermentation.

1. INTRODUCTION

Lignocellulosic biomass, such as cocksfoot grass and wheat straw, is attractive feedstocks that have potential to produce considerable amounts of bioethanol as a promising alternative fuel for transportation sector. The bioethanol produced from these biomass resources are clean and renewable and thus, present a sustainable form of energy alternatives to fossil fuels (Nutawan et al., 2010). The lignocellulosic biomass is the most abundant biomass on earth, and its sources range from trees to agricultural residues (Agbogbo and Wenger, 2007). These lignocellulosic biomass resources are highly complex, mainly comprised of cellulose, hemicellulose, and lignin that are not directly accessible for microbial degradation (Chen and Qiu, 2010). Cellulose and hemicelluloses are both carbohydrates polymers build-up by long chains of sugar monomers, which can be fermented into ethanol after pretreatment and hydrolysis by microbial action (Petersson et al., 2007).

Production of bioethanol as a liquid fuel from lignocellulosic biomass creates technical challenges, such as a need for pretreatment (Petersson et al., 2007). Since lignocellulosic biomass are only partially degradable in their native form, various techniques of mechanical, chemical and biological means have been developed to disrupt the lignocellulose structure and make it susceptible to enzymatic and microbial action (Xuejun and Yoshihiro, 2005), such as wet explosion (Rana et al., 2012), wet oxidation (Schmidt and Thomsen, 1998), dilute acid hydrolysis (Saha et al., 2005a), and steam explosion (Ballesteros et al., 2006). To achieve high overall ethanol yields, the pretreatment should maximize the down-stream enzymatic hydrolysis, and be effective for treatment of biomass at high dry matter concentrations (Georgieva et al., 2008). Wet oxidation and dilute acid as a pretreatment method has been successfully implemented in laboratory and pilot scale plants for treating several lignocellulosic biomass resources for the production of bioethanol and bio-based products (McGinnis et al., 1983; Saha et al., 2005a). Martin et al. (2008) reported approximately 94% conversion efficiency of cellulose to monomeric glucose after enzymatic hydrolysis of cellulose fraction of clover-ryegrass subjected to wet oxidation, showing effective removal of lignin and hemicellulose from cellulose. This study

was, however, done with 6% dry matter concentrations making the results of limited interest for industrial implementation. Taherzadeh and Karimi (2008) further reported that almost 100% hemicellulose removal is possible by dilute acid pretreatment. This method does, however, still need some improvements in order to make it economically interesting for biorefinery processes as the concentration of hexose sugars coming from the pretreatment stream needs to be increased as well as the current need for detoxification (Kuhad et al., 2010). Furthermore, the pretreatment process cost should be reduced by operating at high dry matter concentrations, which will increase the final ethanol concentrations and, at the same time, reduce the reactor volume and minimize wastewater generation (Georgieva et al., 2008).

Under pretreatment, lignocellulosic biomass is converted from its native form, in which it is highly recalcitrant to cellulose enzyme systems, into a form for which cellulose hydrolysis is effective (Hendriks and Zeeman, 2009). The main purpose of pretreatment is to break the lignocellulosic structure (Kumar et al., 2009), and to reduce the cellulose crystallinity with low loss of sugar compounds (Karimi et al., 2006; Zheng et al., 2009). At the same time, the formation of degradation products that inhibit the microbial activities during ethanol fermentation should be kept low (Mosier et al., 2005).

In this present study, wet explosion (WEx) was applied to cocksfoot grass at high dry matter concentration w/w of 25% based on previous investigations on wet explosion with addition of dilute sulfuric acid at a lower dry matter concentration w/w of 14% (Njoku et al., 2012). The effects of different combinations of WEx pretreatment parameters on the biomass composition and enzymatic hydrolysis of the treated substrate was evaluated. Subsequently, the conversion of hexose sugars of cocksfoot grass into ethanol by *Saccharomyces cerevisiae* was investigated.

2. MATERIAL AND METHODS

2.1. Raw material

Cocksfoot grass from the island of Bornholm, Denmark, was harvested in August. The biomass sample was air-dried and hammer milled to a particle size of 2-3 mm and stored in

plastic bags at room temperature prior to pretreatment. A portion of the raw biomass was ground in a coffee grinder (Butler UGS, China) to pass a 1 mm screen and used for chemical composition analysis. Dry matter contents (DM), volatile solid contents (VS), and ash were determined according to the procedure described by the American Public Health Association (APHA, 1992).

The content of total carbohydrates (cellulose and hemicellulose), and Klason lignin in the raw biomass was determined by strong acid hydrolysis according to the procedure developed by the National Renewable Energy Laboratory (Sluiter et al. 2008a). Subsequently, sugar analysis (glucose and xylose) was performed by high performance liquid chromatography (HPLC) refractive index (RI) equipped with an Aminex HPX-87P column (Bio-Rad Laboratories, CA, USA) at 83 ^{o}C with deionized water (Thermo Scientific, Barnstead Nanopure, IA, USA) as an eluent with a flow rate of 1.0 mL/min. Prior to HPLC analysis, samples were centrifuged at 4000 *g* for 10 min, and filtered through a 0.45 μm syringe filter.

The VS content of the raw biomass found as the difference between the total VS value and the sum of the carbohydrate fractions analyzed in the raw biomass was referred to as "other organic matter", which is the sum of proteins, fats and volatile compounds.

2.2. Wet explosion pretreatment

The wet explosion (WEx) pretreatment was performed batch-wise by suspending the raw cocksfoot grass with tap water to reach a dry matter concentration w/w of 25% in a 10 L high-pressure reactor constructed at the Center for Bioproducts and Bioenergy, Washington State University, USA (Rana et al., 2012). The reactor was equipped with a gas/liquid inlet for injection of dilute sulfuric acid or oxygen pressure, and a continuous stirrer (2000 rpm). The reactor was heated by a water jacket connected to a heat exchanger controlled by an oil heater. The temperature and pressure inside the reactor were monitored by two temperature sensors and one pressure sensor both mounted in the headspace and in the bottom of the reactor. The pretreatment was carried out at different temperatures based on the previous optimization trials with the following conditions: temperature (160-190 ^{o}C), oxygen pressure O_2 (6 bars) and dilute sulfuric acid concentration (0.2%) at a retention time

of (15 min). The conditions of pretreatment trials are presented in Table 1. The acid concentration or oxygen pressure was injected into the pretreatment reactor after the desired temperature was reached. After the treatment, the biomass was flashed into a 100 L flash tank connected to the reactor, resulting in a sudden drop in temperature and pressure.

The resulting biomass slurry from the pretreatment was separated into liquid and solid fractions by vacuum filtration. The solid fraction was washed thoroughly with milliQ water and stored in a freezer (-16 ^{o}C) prior to compositional analysis and further processing. The separated liquid fraction was stored at 5 ^{o}C for further analyses.

Table 1. Process conditions used for WEx pretreatment of cocksfoot grass.

Condition	Temp. (^{o}C)	R/T[a] (min)	Oxygen (bar)	Acid concen.[b] [%]
O2-A	160	15	6	-
O2-B	170	15	6	-
O2-C	180	15	6	-
AC-D	170	15	-	0.2
AC-E	180	15	-	0.2
AC-F	190	15	-	0.2

[a]Retention time. [b]Acid concentration.

2.3. Analysis of the solid and liquid fraction

The washed solid fraction (portion) obtained after separation of the WEx slurry was dried in an incubator at 38 ^{o}C for 12 h before compositional analysis. The dried solids were ground in a coffee grinder (Butler UGS, China) to pass a 1 mm screen before chemical compositional analysis. The content of total carbohydrates (cellulose and hemicelluloses), and Klason lignin in the separated solid fractions was determined by strong acid hydrolysis as previously described (Sluiter et al., 2008a).

Carbohydrates in the liquid fractions (filtrate) after WEx pretreatment were both polymers and oligomers together with small amounts of monomers, and hence the samples were hydrolyzed using 4% (w/w) sulfuric acid at 121 ^{o}C for 10 min to determine the total xylose, arabinose, and glucose concentration in the filtrate. The analysis was determined according to the National Renewable Energy Laboratory protocol (Sluiter et al., 2008b). Glucose, xylose, arabinose present in the liquid fractions were quantified by HPLC as

described in Section 2.1, and furfural, hydroxymethylfurfural (HMF), and acetic acid also present in the liquid fractions were measured by HPLC refractive index (RI) equipped with an Aminex HPX-87H column (Bio-Rad Laboratories, CA, USA) at 60 ^{o}C with 4 mM H_2SO_4 as an eluent with a flow rate of 0.6 mL/min.

2.4. Enzymatic convertibility of cellulose in the solid fraction

The release of hydrolysable cellulose by wet explosion pretreatment was analyzed by the sugars released after enzymatic hydrolysis using a commercial enzyme mixture (Cellic CTec2), kindly provided by Novozymes North America (Franklinton, USA). The enzymatic convertibility of the separated WEx solid fraction was carried out at 10% DM with 0.05 M succinate buffer (pH 5.0). The experiments were performed in duplicates in 2 mL Eppendorf tubes filled with 1.5 mL of hydrolysis media and an enzyme dosage of 20 mg-EP/g-VS (EP = enzyme protein) for all samples. The hydrolysis mixture was incubated for 72 h at 50 ^{o}C in a thermomixer shaker at 1400 rpm. The reaction was stopped by heating the solution to 100 ^{o}C for 10 min, mixed by vortexing, and centrifuged for 8 min at 3600 *g*. The concentration of glucose, xylose, and arabinose in the hydrolyzate was quantified by HPLC as previously described in Section 2.1.

2.5. Ethanol fermentation process

The hexose sugars from the WEx separated solid fraction was fermented to ethanol through simultaneous saccharification and fermentation (SSF) by *Saccharomyces cerevisiae* (Thermosacc®, USA). The *S. cerevisiae* inoculum culture medium was prepared aseptically in 250-mL shaking flask covered with cotton stopper with 100 mL medium containing 10 g/L yeast extract, 20 g/L peptone and 20 g/L D-glucose, and incubated on a rotary shaker at 160 rpm and 32 ^{o}C for 24 h. All media were sterilized by autoclaving at 121 ^{o}C for 30 min. The cells were harvested by centrifugation, and the pellet was collected for SSF fermentation to a final optical density (OD) of 0.5 measured at (600 nm), corresponding to a cell concentration of around 0.9 g/L. Presaccharification (liquefaction) and SSF was performed under anaerobic condition in sterile 250 mL shaking flasks with 100 mL fermentation media as the working volume.

Presaccharification of the material was performed at 50 ^{o}C for 6 h at an enzyme dosage of 10 mg-EP/g-VS using Cellic CTec2, adjusted to pH 4.8 with 1 M citrate buffer in order to liquefy the solid fractions and ensure a proper substrates mixing. After presaccharification, the shaking flasks were then cooled to room temperature and supplemented with second batch of enzyme mixture (Cellic CTec2) at an enzyme dosage of 10 mg-EP/g-VS, inoculated with the yeast cells under aseptic condition and the pH was maintained at 4.8 by addition of 0.05 M citrate buffer solution. The shaking flasks were sealed with bubbler airlocks filled with water and incubated on a rotary shaker at 150 rpm and 32 ^{o}C for 168 h. Samples were withdraw at regular intervals for sugar and ethanol concentrations and were determined by HPLC as described previously in Section 2.1. All the experiments were performed in duplicates at the same initial cell concentration.

2.6. Calculations

The recovery of sugars in the solid or filtrate during the WEx pretreatment process was calculated according to Eq. (1). The yields after WEx and enzymatic hydrolysis of the solid fractions were calculated according to Eq. (2) for glucose released and (3) for pentoses released (xylose and arabinose): where glucose_{EH} is the mass of glucose released after enzymatic hydrolysis of cellulose in the solid fraction and pentoses_{LF} is the mass of pentose sugars (xylose and arabinose) released after WEx pretreatment in the liquid fraction. The ethanol yield (Y_{EtOH}) was calculated by dividing the total amount of ethanol produced by the initial dry weight of treated cocksfoot grass. The percent theoretical (stoichiometric) ethanol yield ($\%Y_{EtOH}$) was calculated according to Eq. (4): where 1.11 is the stoichiometric conversion factor of cellulose to equivalent glucose and 0.51 is the theoretical ethanol yield (in grams) generated per 1 g of glucose, Hatzis et al. (1996). This yield is always less than 100% as part of the sugars is converted to cell mass and by-products by the organisms.

$$\text{Recovery (\%)} = \frac{\text{sugar in solid} + \text{sugar in filtrate} / 1.11 \text{ or } 1.14}{\text{sugar in raw material}} \cdot 100 \qquad (1)$$

$$\text{Hydrolysis yield}_{\text{glucose}}\ (\%) = \frac{\text{glucose}_{\text{EH}}\ / 1.11}{\text{cellulose in raw material}} \cdot 100 \qquad (2)$$

$$\text{Hydrolysis yield}_{\text{pentoses}}\ (\%) = \frac{\text{pentoses}_{\text{LF}}\ / 1.14}{\text{hemicellulose in raw material}} \cdot 100 \qquad (3)$$

$$Y_{\text{EtOH}}\ (\%) = \frac{\text{produced EtOH(g)}}{\text{cellulose in raw material} \cdot 1.11 \cdot 0.511} \cdot 100 \qquad (4)$$

3. RESULTS AND DISCUSSION

3.1. Raw material composition

The chemical composition of raw material shows that it has a high concentration of cellulose and contains a fair amount of hemicelluloses (36 and 24 g per 100 g DM, respectively), which is comparable or higher than the amounts found in wheat straw (33.9 g and 23.0 g per 100 g DM for cellulose and hemicellulose, respectively) by Thomsen et al. (2006). The dry matter content is approximately 93%, which makes it attractive as ethanol feedstock. The lignin content is relatively higher than the content of other organic matter, which is the sum of non-analyzed organic matter like pentoses other than xylose and arabinose, proteins and fats in biomass.

3.2. WEx pretreatment and recovery of carbohydrates and lignin

As shown in (Fig. 1), the recovery of cellulose, hemicellulose and lignin varied with the conditions of the WEx pretreatment process. The WEx pretreatment was expected to fractionate the lignocellulosic material into a solid fraction containing mainly cellulose and lignin, and a liquid fraction enriched with solubilized hemicelluloses mainly present as C5 monomers, and low molecular lignin fragments in dissolved form.

The most efficient cellulose and hemicelluloses recovery of 99 and 97%, respectively, was found for WEx pretreatment condition *O2-A* (160 ^{o}C, 15 min, 6 bar O_2), higher than previously reported for high dry matter concentrations. The lowest recovery of cellulose at

94% was found for WEx pretreatment conditions *AC-F* and *O2-C* (190 ^{o}C, 15 min, 0.2% sulfuric acid and 180 ^{o}C, 15 min and 6 bar O_2). The same conditions (*O2-C* and *AC-F*) achieved the lowest hemicelluloses recovery at 55 and 61%, respectively, (Fig. 1). Generally, we found high recovery of cellulose and hemicelluloses under WEx pretreatment at temperature below 180 ^{o}C, whereas, applying the process temperature above 170 ^{o}C significantly degraded significant parts of the hemicellulose sugars, especially, WEx pretreatment temperature at 180 ^{o}C and 6 bars O_2 resulting in loss of 45% of the hemicellulose sugars and thus, gave the lowest recovery of the hemicellulose fractions.

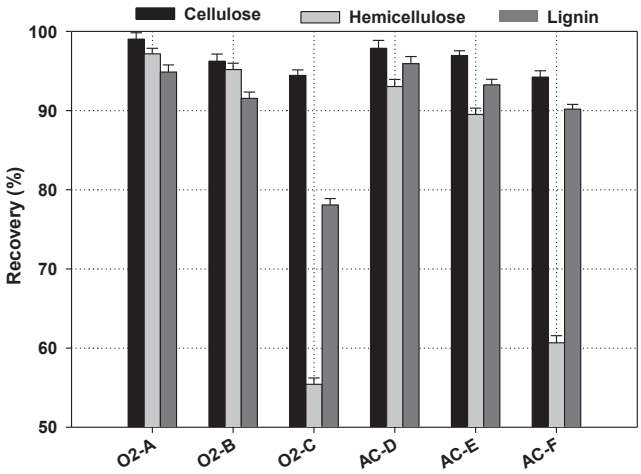

Fig. 1. Recovery of polysaccharides (cellulose and hemicellulose) and lignin after WEx pretreatment at different conditions (error bars represent standard deviations of duplicates measurements).

The recovery of cellulose and hemicelluloses found for WEx pretreatment with the lowest severe condition (*O2-A*) is higher than what has been previously reported with lignocellulosic biomass subjected to various pretreatment methods. Petersson et al. (2007) achieved 92 and 69% recovery of cellulose and hemicellulose sugars from faba bean straw under wet oxidation pretreatment at 195 ^{o}C for 15 min, 2 g/L Na_2CO_3 and 12 bar O_2. WEx

pretreatment condition (*AC-D*) achieved cellulose and hemicellulose recovery of 97% and 93%, respectively; which is higher than the recovery of 93 and 72% for cellulose and hemicellulose, respectively, reported by Georgieva et al. (2008) after subjecting wheat straw to wet explosion pretreatment at 180 ^{o}C, 35% (*v/v*) hydrogen peroxide for 15 min. Operational parameters for pretreatment must be tailored to the specific biomass structural composition. It was evident in this study that the combination of pretreatment temperature, dilute acid and/or oxygen pressure was the most crucial factors for gaining high recovery of sugars for cocksfoot grass biomass.

The high recovery of cellulose among the tested pretreatment conditions reveals an efficient separation of the cellulose from lignin and low degradation of cellulose to other products, such as HMF during the WEx pretreatment process. On the other hand, hemicellulose recovery varied in a wider range among the pretreatment conditions than cellulose, and the conditions with higher pretreatment severity significantly degraded hemicellulose sugars, and accordingly achieved below 90% hemicellulose recovery.

The highest degree of lignin solubilization was achieved in pretreatment condition *O2-C*, due to the combination of high pretreatment temperature and oxygen pressure as an oxidizing agent. This is in agreement with the fact that lignin tends to undergo both cleavage and oxidation during wet oxidation which facilitates the solubilization of lignin during pretreatment with oxidizing agent (Taherzadeh and Karimi, 2008). Generally, the lignin recovery in the solid fraction among the pretreatment conditions was above 70% (Fig. 1). The highest recovery of lignin in the solid fraction was obtained under WEx pretreatment condition *AC-D* showing approximately 96% lignin recoveries. As lignin recovery in the solid fraction after pretreatment is affected by solubilization and depolymerization of sugars (Sassner et al., 2008), lignin may be degraded to low molecular lignin compounds i.e. phenolic compounds under harsh pretreatment conditions (Petersen et al., 2009). The high recovery of lignin in the solid fraction indicate that only a small part of lignin was solubilized under the WEx conditions with low pretreatment temperatures and, hence, the formation of low molecular lignin compounds was low.

3.3. Enzymatic hydrolysis of cellulose in the solid fraction and yield of sugars in the liquid fraction after pretreatment

The enzymatic accessibility of the cellulose was investigated for all the pretreated solid fractions under WEx conditions as this is one of the most important factors to evaluate the efficiency of the pretreatment for the production of bioethanol from hexose sugars (Varga et al., 2003).

Enzymatic convertibility of cellulose in solid fraction pretreated at high dry matter concentration (25%) varied between 88 and 98% for the different pretreatment conditions (Fig. 2A). The highest convertibility of cellulose in the solid fraction, 98%, was achieved for the WEx condition *AC-E* (180 ^{o}C, 15 min, 0.2% sulfuric acid), and this is in accordance with that dilute acid pretreatment is not effective in lignin solubilization, but it can disrupt lignin and increase the cellulose susceptibility to enzymatic action (Taherzadeh and Karimi, 2008). However, good results were also achieved for WEx pretreatment conditions *O2-C* and *AC-F* (180 ^{o}C, 15 min, 6 bar O_2 and 190 ^{o}C, 15 min, 0.2% sulfuric acid) resulting in a 93% glucose yield. The lowest glucose yield was obtained at WEx condition *O2-A*, although, this condition gave the highest overall cellulose and hemicellulose recovery. This result support the idea that the optimum conditions for the highest sugar recovery do not necessary mean the most effective conditions for enzymatic conversion of cellulose fractions to sugar monomers (Taherzadeh and Karimi, 2008). It has been previously reported that combining high pretreatment temperature and prolonging the treatment time could increase the efficiency of hydrolysis of cellulose materials (Taherzaden and Niklasson, 2004), and a high pretreatment temperature is more suitable for achieving high glucose yields (Sørensen et al., 2008). In comparison, Martin et al. (2008) reported 93.6% cellulose conversion of the solid fraction after wet oxidation pretreatment of clover-ryegrass mixtures at 195 ^{o}C, 10 min, and 1.2 MPa. It has been previously demonstrated that pretreatment of lignocellulosic biomass at high dry matter concentration is an ideal method to improve ethanol yields; Lu et al. (2009) found 63.7% glucose yield after enzymatic hydrolysis of pretreated rapeseed straw under sulfuric acid-catalyzed hydrothermal pretreatment at 180 ^{o}C, 1% sulfuric acid, 20% solids content for 10 min. Generally, all the pretreatment conditions tested in our study achieved good glucose yields above 88%, which

is higher than previously reported for biomass pretreatment at high dry matter concentrations as used in our study.

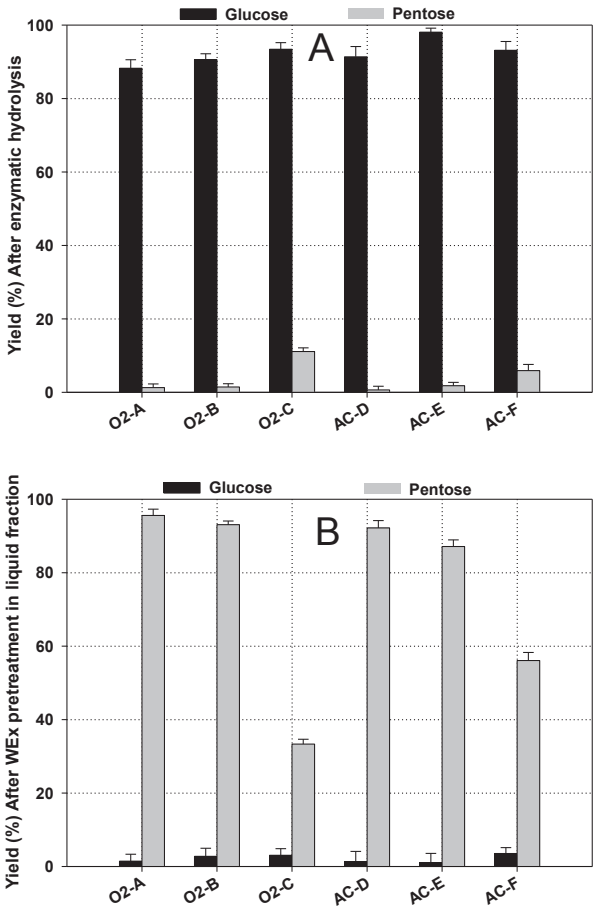

Fig. 2A. Yield of glucose and pentoses after enzymatic hydrolysis of solid fractions. **(B).** Yield of glucose and pentoses in the liquid fraction after WEx pretreatment. Average values and standard deviation reported for duplicates analysis.

The pentose sugars yield (xylose and arabinose) are presented in (Fig. 2B). The highest solubilized pentoses was found under the less severe conditions; pretreatment condition

O2-A (160 ^{o}C, 15 min, 6 bar O_2) gave approximately 96% yield of pentose sugars. Furthermore, pretreatment conditions *O2-B* and *AC-D* (170 ^{o}C, 15 min, 6 bar O_2 and 170 ^{o}C, 15 min, 0.2% sulfuric acid) achieved a yield of 93 and 92%, respectively, which is higher than 68% total pentoses yield achieved by Thomsen et al. (2008) for wet oxidation pretreatment of wheat straw at 190 ^{o}C, H_2O_2 and 6 min. The pretreatment conditions (*O2-C* and *AC-F*) significantly degraded a large fraction of the pentose sugars and thus, achieved the lowest yield of around 33 and 56%, respectively, showing that lower pretreatment temperature is more advantageous for maximizing pentose yields. The extraction of hemicellulose sugars in the liquid fraction were more pronounced in lower pretreatment severity, especially, the pretreatment at temperature of 160 ^{o}C. It is obvious that the release of hexose and pentose sugars needs different pretreatment severity, as the conditions where the best hexose sugars were obtained will convert free pentose sugars found in the solution.

The total yield of sugars (hexose and pentose) is presented in Table 2. Total yield of sugars was 526.3-542.0 g/kg DM, corresponding to 88-91% of theoretical in most of the WEx pretreatment conditions, but was lower (475 g/kg DM, corresponding to 80% of total yield of sugars) for the most severe tested pretreatment conditions *O2-C* and *AC-F* (180 ^{o}C, 15 min, 6 bar O_2 and 190 ^{o}C, 15 min, 0.2% sulfuric acid) owing to the high degree of sugar degradation to other products such as HMF and furfural in these conditions. For an economically viable process, the best condition was identified as *O2-B* with respect to low formation of by-products and yield of both C5 and C6 sugars. Considering the overall process economy with regards to process temperature (energy consumption), condition *O2-A* can be identified as the best WEx pretreatment condition for achieving both high total yield of sugars (526 g/kg DM, which amounts to 89%) with moderate energy input. Pretreatment conditions *AC-D*, *O2-B* and *AC-E* gave slightly higher total sugars yields (1-2%) (528, 533, and 542 g/kg DM, respectively) as shown in Table 2, but here the temperature was increased 10 and 20 ^{o}C, respectively. It is therefore, crucial to consider all parameters before the pretreatment conditions are selected. Pretreatment reactors for dilute acid pretreatment will need to be made of acid-resistant materials which will add extra cost compared to reactors operated with oxygen addition.

Table 2. Total yield of sugars among the WEx conditions based on sugar release from C6 and C5 fractions after WEx pretreatment and enzymatic hydrolysis.

WEx conditions	Total Yield of Sugars	
	(g/kg DM)	(%)
O2-A	526.3 ± 0.14	88.54
O2-B	533.2 ± 0.17	89.70
O2-C	474.7 ± 0.11	79.86
AC-D	528.3 ± 0.08	88.88
AC-E	542.0 ± 0.15	91.18
AC-F	475.7 ± 0.06	80.03

Errors presented here were standard deviation of duplicate experiments.

3.4. Formation of degradation products

The formation of acetic acid was higher among the WEx pretreatment conditions (Fig. 3), as this was the main degradation products measured in the liquid fraction of the pretreated material. The highest formation of acetic acid (4.59 g/100 g DM) was found for pretreatment condition *O2-C*, followed by pretreatment condition *AC-F*, with a level of 2.77 g/100 g DM. The formation of acetic acid during pretreatment has been reported elsewhere in the literature to be associated with high pretreatment temperature and longer treatment time (McGinnis et al., 1983). This is evident as the pretreatment conditions (*O2-C* and *AC-F*) for oxygen and dilute sulfuric acid, respectively, gave the highest formation of acetic acid, which is further confirmed by the lower yield of hemicellulose sugars associated with the above-mentioned conditions. Depending on the process severity, carboxylic acids (mainly acetic acid), furan derivatives (furfural and 5-hydroxymethyl furfural-HMF), and phenolic compounds are generated during pretreatment of lignocellulosic biomass, and are considered potential fermentation inhibitors (Saha, 2004; Klinke et al., 2004). At more severe pretreatment conditions, hemicellulose sugar monomers are degraded to furfural while HMF is formed from hexose degradation, and phenolic compounds are liberated from partial breakdown of lignin (Palmqvist and Hahn-Hägerdal, 2000).

Furfural and HMF formation was low in most of the pretreatment conditions. The highest concentration of furfural was found under condition (*O2-C*), around 2 g/100 g DM

(Fig. 3). HMF formation was the lowest among the two by-products. The formation of furfural and HMF in the liquid fraction during pretreatment is a result of the dehydration of pentose and hexose sugars, respectively, under thermal and acidic conditions (Martin et al., 2007). The results presented in (Fig. 3) shows that the most severe pretreatment conditions achieved the highest concentrations of the measured by-products in the WEx liquid fraction. This is in agreement with previous investigations where the production of these compounds increases with higher pretreatment temperatures (Mosier et al., 2005), and these compounds not only reduces the sugar yield, but can also inhibit the fermentation process. The formation of the byproducts found in the current study is in good agreement with Martin et al. (2007), who reported relatively the same amounts of these byproducts for sugarcane bagasse subjected to wet oxidation with pretreatment conditions (195 ^{o}C, 15 min, and alkaline pH) gave 9.21 g/100 g material for carboxylic acids (acetic acid and glycolic acid), 0.53 g/100 g material for furfural, and 0.07 g/100 g material for HMF, respectively, compared to our highest found values of (g/100 g DM): acetic acid, 4.59; furfural, 2.26; and HMF, 0.53.

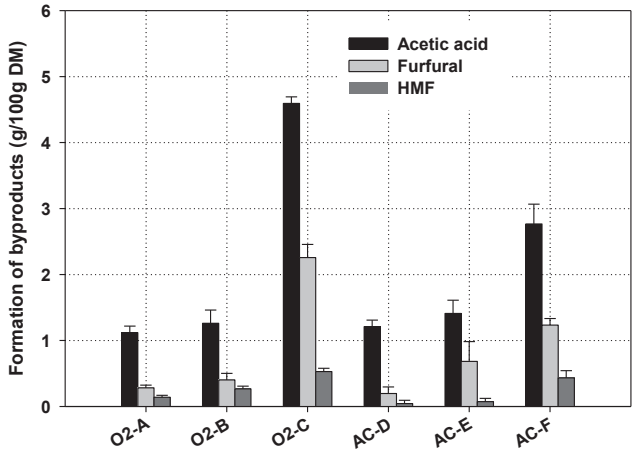

Fig. 3. Formation of by-products in liquid fraction after WEx pretreatment. Results are average of duplicates and error bars represent standard deviations.

3.5. Simultaneous saccharification and fermentation (SSF)

The separated solid fractions enriched in cellulosic sugars after pretreatment were subjected to an SSF process with simultaneous hydrolysis and fermentation to ethanol using *S. cerevisiae*. Prior to the SSF process, the substrate was liquefied for 6 h enabling sufficient mass transfer in the fermentation broth and, thus decrease the viscosity of the material inside the fermentation medium. The yeast *S. cerevisiae* exhibit high tolerance towards fermentation inhibitors and is the most efficient organism for glucose fermentation (Georgieva et al., 2008). The ethanol and glucose concentration profiles during the fermentation are presented in (Fig. 4A and B), as the concentration of glucose decreased to nearly 2 g/L, the ethanol concentrations rapidly increased from zero to around 27 g/L during the first 72 h of fermentation (Fig. 4A). This is comparable with what have been reported elsewhere in the literature (Bertilsson et al., 2009). After 72 h, glucose was still released from the cellulose fraction at a very low rate (Fig. 4B); this was observed by a slight increase in ethanol concentration which was kept stable after 120 h. A lag phase was not observed during the course of fermentation, probably due to subsequent washing of the solid fractions prior to SSF process, thereby reduces the risk of containing fermentation inhibitors at inhibitory level.

An ethanol concentration of about 33.14 g/L was achieved under the pretreatment condition *AC-E*, corresponding to a yield of 250.1 mL/kg DM (97% of the theoretical maximum yield), and this is the highest achieved ethanol yield in our study for C6 conversion by *S. cerevisiae* (Table 3).

Surprisingly, condition *AC-F* (190 ^{o}C, 15 min, 0.2% sulfuric acid) achieved ethanol yield of 237.3 mL/kg DM (92% of the theoretical maximum value) lower than condition *AC-E* (180 ^{o}C, 15 min, 0.2% sulfuric acid), showing that this pretreatment condition has partially degraded some of the cellulose sugars, presumably due to the severely destruction of the cellulose crystalline structure. On the other hand, condition *O2-C* (180 ^{o}C, 15 min, 6 bar O_2) also gave a lower ethanol yield compared to condition (*AC-E*) at the same pretreatment temperature. It has been previously documented that pretreatment at elevated temperature combined with oxygen pressure can partly degrade cellulose fractions to other byproducts due to oxidation reaction that associated with oxygen (Taherzadeh and Karimi,

2008). The 97% yield of the theoretical maximum value found in this study is in good agreement with previous study on SSF process with *S. cerevisiae* (Thomsen et al., 2006).

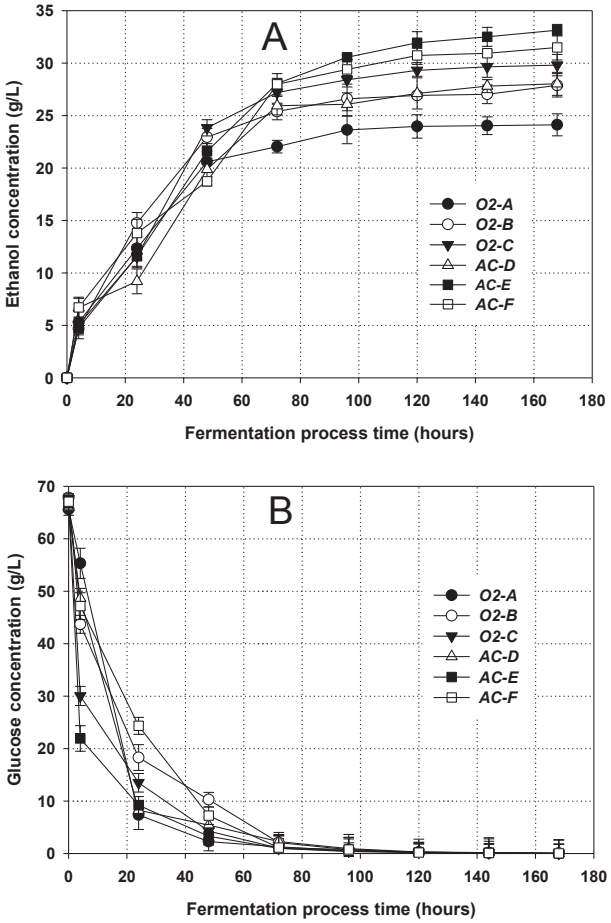

Fig. 4**A**. Time course of ethanol formation during simultaneous saccharification and fermentation (SSF) process of cellulose fraction by *S. cerevisiae* incubated over 168 h, 150 rpm at 32 ^{o}C and pH 4.8. (**B**) Time course of glucose utilization during ethanol fermentation over 168 h. Values are means of duplicate experiments.

The lowest achieved ethanol yield of 179.7 mL/kg DM (70% of the theoretical maximum) was found under the less severe condition (*O2-A*). The reason could be that the cellulose structure had not been significantly altered under this condition and, hence, could not easily be hydrolyzed to cellulose for simultaneously conversion into ethanol by *S. cerevisiae*. However, good results were also obtained from other conditions (*O2-B* and *AC-D*), around 80% of the theoretical maximum possible yield. This result is higher or comparable to ethanol yields reported for wheat straw and clover-ryegrass mixtures solid fractions fermented to ethanol by *S. cerevisiae* (Petersen et al., 2009; Martin et al., 2008).

Table 3. Summary of the fermentation results from SSF process among the WEx conditions.

WEx conditions	Final ethanol concentration (g/L)	Ethanol yield (mL/kg-DM)	% of theoretical Yield
O2-A	24.12 ± 0.05	179.70 ± 0.09	69.9
O2-B	27.89 ± 0.07	201.00 ± 0.03	78.2
O2-C	29.80 ± 0.02	225.50 ± 0.13	87.7
AC-D	28.02 ± 0.04	211.30 ± 0.16	82.2
AC-E	33.14 ± 0.17	250.10 ± 0.06	97.3
AC-F	31.49 ± 0.14	237.30 ± 0.04	92.3

Errors presented here were standard deviation of duplicate experiments. Fermentations were performed at 32 ^{o}C in a rotary shaker incubator at 150 rpm over 168 h at pH 4.8.

4. CONCLUSIONS

Our present investigation on cocksfoot grass revealed that wet explosion is a promising pretreatment method for producing high sugar and ethanol yields. The highest monomeric C6 sugars release from the cellulose fraction after WEx pretreatment and enzymatic hydrolysis was attributed to condition *AC-E* (180 ^{o}C, 15 min, 0.2% sulfuric acid), on the other hand, the release of hemicellulose sugars in the liquid fraction was more pronounced under WEx condition *O2-A* (160 ^{o}C, 15 min, 6 bar O_2) where a significant higher yield was achieved. From these results, we found that disrupting the biomass complex structures using oxygen pressure combined with temperatures around 180 ^{o}C results in destruction of

both hexose and pentose sugars, where significant amount of sugars were degraded to by-products such as acetic acids. This is evident as the main reactions for oxidative pretreatment at high temperatures are the formation of acids. The wet explosion pretreatment condition (*AC-E*) at 180 ^{o}C achieved the highest ethanol yield of 250 mL/kg DM (97% of theoretical) from the cellulose fraction subjected to SSF process, whereas, the WEx condition (*O2-C*) at 180 ^{o}C gave slightly lower ethanol yield of 226 mL/kg DM. It is quite obvious from this present study that the release of hexose and pentose sugars have very different dynamics and, therefore, pretreatment process parameters should be tailored to the specific biomass compositional structures and with a view to all the potential sugars which can be produced.

ACKNOWLEDGMENTS

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REFERENCES

Agbogbo, F. K., Wenger, K. S., 2007. Production of ethanol from corn stover hemicellulose hydrolyzate using *Pichia stipitis*. Ind. Microbiol. Biotechnol. 34, 723-727.

American Public Health Association (APHA), American Water Works Association (AWWA), and Water Environment Federation (WAE), 1992. Standard Methods for the Examination of Water and Waste Water. (Ed. Greenberg, A. E., Clesceri, L. S., Trussel, R. R.), 18th edition.

Ballesteros, I., Negro, M. J., Oliva, J. M., Cabanas, A., and Ballesteros, M., 2006. Ethanol produced from steam-explosion pretreated wheat straw. Appl. Biochem. Biotechnol. 129-132, 496-508.

Bertilsson, M., Olofesson, K., Liden, G., 2009. Prefermentation improves xylose utilization in simultaneous saccharification and fermentation of pretreated spruce. Biotechnology for Biofuels 2:8, 1754-6834.

Chen, H., Qiu, W., 2010. Key technologies for bioethanol production from lignocellulose. Biotechnol. Advances 28, 556-562.

Georgieva, T. I., Hou, X., Hilstrøm, T., Ahring, B. K., 2008. Enzymatic hydrolysis and ethanol fermentation of high dry matter Wet-Exploded wheat straw at low enzyme loading. Appl. Biochem. Biotechnol. 148, 35-44.

Hatzis, C., Riley, C., Philippidis, G. P., 1996. Detailed material balance and ethanol yield calculations for biomass-to-ethanol conversion process. Appl. Biochem. Biotechnol. 96, 0273-2289.

Hendriks, A. T. W. M., Zeeman, G., 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresource Technology 100, 10-18.

Karimi, K., Emtiazi, G., Taherzadeh, M. J., 2006. Ethanol production from dilute–acid pretreated rice straw by simultaneous saccharification and fermentation with *Mucor indicus, Rhizopus oryzae, and Saccharomyces cerevisiae.* Enzyme and Microbial Technol. 40, 138-144.

Klinke, H. B., Thomsen, A. B., Ahring, B. K., 2004. Inhibition of ethanol–producing yeast and bacteria by degradation products produced during pre–tretreatment of biomass. Appl. Microbiol Biotechnol. 66, 10-26.

Kuhad, R. C., Gupta, R., Khasa Y. P., Singh, A., 2010. Bioethanol production from *lantana camara* (red sage): Pretreatment, saccharification and fermentation. Bioresource Technology 101, 8348-8354.

Kumar, P., Barrett, D. M., Delwiche, M. J., and Stroeve, P., 2009. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind. Eng. Chem. Res. 48, 3713-3729.

Lu, X., Zhang, Y., Angelidaki, I., 2009. Optimization of H_2SO_4-catalyzed hydrothermal pretreatment of rapeseed straw for bioconversion to ethanol: Focusing on pretreatment at high solids content. Bioresource Technology 100, 3048-3053.

Martin, C., Klinke, H. B., Thomsen, A. B., 2007. Wet oxidation as a pretreatment method for enhancing the enzymatic convertibility of sugarcane bagasse. Enzyme and Microbial Technology 40, 426-432.

Martin, C., Thomsen, M. H., Hauggaard-Nielsen, H., Thomsen, A. B., 2008. Wet oxidation pretreatment, enzymatic hydrolysis and simultaneous saccharification and fermentation of clover-ryegrass mixtures. Bioresource Technology 99, 8777-8782.

McGinnis, G. D., Wilson, W. W., Mullen, C. E., 1983. Biomass pretreatment with water and high-pressure oxygen. The wet-oxidation process. Ind. Eng. Chem. Prod. Res. Dev. 22, 352-357

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M., Ladisch, M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technology 96, 673-686.

Njoku, S. I., Uellendahl, H., Ahring, B. K., 2012. Tailoring wet explosion process parameters for the pretreatment of Cocksfoot grass for high sugar and ethanol yields. Manuscript submitted for publication.

Nutawan, Y., Phattayawadee, P., Pattranit, T., and Mohammad-Naghi, E., 2010. Bioethanol produced from rice straw. Energy Research Journal 1, 26-31.

Palmqvist, E., Hahn-Hägerdal, B., 2000. Fermentation of lignocellulosic hydrolysates II: inhibitors and mechanisms of inhibition. Bioresource Technology 74, 25-33.

Petersen, M., Larsen, J., Thomsen, M. H., 2009. Optimization of hydrothermal pretreatment of wheat straw for production of bioethanol at low water consumption without addition of chemicals. Biomass and Bioenergy 33, 834-840.

Petersson, A., Thomsen, M. H., Hauggaard-Nielsen, H., Thomsen, A. B., 2007. Potential bioethanol and biogas production using lignocellulosic biomass from winter rye, oilseed rape and faba bean. Biomass and Bioenergy 31, 812-819.

Rana, D., Rana, V., Ahring, B. K., 2012. Producing high sugar concentrations from loblolly pine using wet explosion pretreatment. Bioresource Technology 121, 61-67.

Saha, B. C., Iten, L. B., Cotta, M. A., Wu, Y. V., 2005a. Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. Process Biochem. 40, 3693-3700.

Saha, B. C., 2004. Lignocellulose biodegradation and applications in biotechnology. ACS Symp. Ser. 889, 2-34.

Sassner, P., Mårtensson, C. G., Galbe, M., Zacchi, G., 2008. Steam pretreatment of H_2SO_4–impregnated Salix for the production of bioethanol. Bioresource Technology 99, 137-145.

Schmidt, A. S., Thomsen, A. B., 1998. Optimization of wet oxidation pretreatment of wheat straw. Bioresource Technology 64, 139-151.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., and Crocker, D., 2008a. Determination of Structural Carbohydrates and Lignin in Biomass. Laboratory Analytical Procedure, Technical Report NREL/TP-510-42618.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., and Templeton, D., 2008b. Determination of Sugars, Byproducts and Degradation Products in Liquid Fraction Process Samples. Laboratory Analytical Procedure, Technical Report NREL/TP-510-42623.

Sørensen, A., Teller, P. J., Hilstrøm, T., Ahring, B. K., 2008. Hydrolysis of miscanthus for bioethanol production using dilute acid presoaking combined with wet explosion pre-treatment and enzymatic treatment. Bioresource Technology 99, 6602-6607.

Taherzadeh, M. J., Karimi, K., 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review. Int. J. Sci. 9, 1621-1651.

Taherzadeh, M. J., Niklasson, C., 2004. Ethanol from lignocellulosic materials: pretreatment, acid and enzymatic hydrolysis, and fermentation. ACS Symp. Ser. 889, 49-68.

Thomsen, M. H., Thygesen, A., Jørgensen, H., Larsen, J., Christensen, B. H., and Thomsen, A. B., 2006. Preliminary results on optimization of pilot scale pretreatment of wheat straw used in coproduction of bioethanol and electricity. Appl. Biochem. And Biotechnol. 126-132, 448-460.

Varga, E., Schmidt, A. S., Reczey, K., Thomsen, A. B., 2003. Pretreatment of corn stover using Wet Oxidation to enhance enzymatic digestibility. Appl. Biochem. and Biotechnol. 104, 0273-2289.

Xuejun, P., Yoshihiro, S., 2005. Fractionation of wheat straw by atmospheric acetic acid process. Bioresource Technology 96, 1256-1263.

Zheng, Y., Pan, Z., Zhang, R., 2009. Overview of biomass pretreatment for cellulosic ethanol. Int. J. Agric. & Biol. Eng. 2, (3): 51-68.

Research paper IV

Production of ethanol from the hemicellulose fraction of cocksfoot grass using *Pichia stipitis*

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Submitted to Sustainable Chemical Processes

Production of ethanol from the hemicellulose fraction of cocksfoot grass using *Pichia stipitis*

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Abstract

In this study, cocksfoot grass (*Dactylis glomerata*), an abundant lignocellulosic biomass was pretreated using different operational parameters using wet explosion (WEx) pretreatment for accessing the bioethanol potential of hemicellulose fraction. Utilization of the hemicellulose liquid hydrolysate to ethanol is essential for economical feasible in cellulosic ethanol processes. Fermentation of the separated hemicellulose liquid hydrolysates obtained after the WEx pretreatment was done with *Pichia stipitis* CBS 6054. The fermentation of WEx liquid hydrolysates from the higher pretreatment severity (180 ^{o}C, 15 min, 87 psi oxygen and 190 ^{o}C, 15 min, 0.2% sulfuric acid) was fully inhibited probable by the presence of higher concentrations of inhibitory compounds such as furfural, HMF and acetic acid. The ethanol yield among other WEx conditions was ranged from 89-158 mL/kg DM, with the highest yield (92% of theoretical maximum value) found for the lower pretreatment severity at 160 ^{o}C, 15 min, 87 psi oxygen.

Keywords: Wet explosion; Lignocellulosic biomass; Cocksfoot grass; Pretreatment; Ethanol fermentation; Inhibitors; *Pichia stipitis*.

1. INTRODUCTION

Increasing global energy requirements and greater environmental awareness have resulted in increasing focus on alternatives to fossil fuels as energy sources. Lignocellulosic biomass such as agricultural residues, forestry waste and municipal solid waste presents a sustainable and renewable source for the production of liquid biofuels such as bioethanol (Taherzadeh and Karimi, 2008). As most often being a by-product from food and feed production, lignocellulosic biomass does not compete with the production of edible crops (Chen and Qiu, 2010; Petersson et al., 2007) and has the potential to be the feedstock for the production of a considerable proportion of transport fuels if cost effective conversion processes are available (Kristensen et al., 2008). The major components in lignocellulosic biomass are cellulose, hemicellulose and lignin. Hemicellulose sugars are the second most abundant carbohydrates in nature and its conversion to ethanol could provide an alternative liquid fuel source for the future (Jeffries, 2006).

Because of the recalcitrance of the lignocellulosic structure to enzymatic attack, pretreatment of the material is necessary to enhance the accessibility of the enzymes to the substrate (Sassner et al., 2008). Various thermal and chemical pretreatment methods as well as combinations of both have been proposed to make lignocellulosic biomass susceptible to enzymatic and microbial conversion (Galbe and Zacchi, 2002; Hendriks and Zeeman, 2009). The resulting slurry from the pretreatment of lignocellulosic biomass contains liquid and solid fractions; the solid fraction mostly contains cellulose and lignin as the major components, while the liquid fraction contains xylose as the main sugar, and small concentrations of other sugars such as glucose and arabinose mainly from hemicellulose liquid hydrolysates. Hence, the optimum utilization of the liquid fractions to ethanol is essential for an economical feasible in biorefinery processes (Agbogbo and Wenger, 2007). However, the liquid fractions often contains inhibitors such as furfural from xylose degradation, hydroxymethylfurfural (HMF) from glucose degradation, carboxylic acids mainly acetic acid from the acetyl group in hemicellulose decomposition, and phenolic compounds from lignin degradation (Agbogbo and Wenger, 2007) and these are considered

to be potential fermentation inhibitors that affect the growth rate of microbes during ethanol fermentations (Zhu et al., 2009).

Microbes such as yeasts and bacteria are essential for the conversion of hemicellulose sugars to ethanol (Jeffries, 2006). *Pichia stipitis* among others is one of the robust xylose-fermenting yeast that has been investigated in many laboratories around the world because of its capability for using pentose sugars beside hexoses with a high ethanol yield (Taniguchi et al., 1997). Moniruzzaman, (1995) reported ethanol yield of 78% theoretical maximum from exploded rice straw hydrolysate fermented to ethanol by *Pichia stipitis* Y-7124. In a similar manner, Zhu et al. (2009) found ethanol yield of around 80% theoretical from steam exploded corn stover acid hydrolyzate fermented to ethanol using *Pichia stipitis* CBS 5776.

The present study investigated ethanol production from hemicellulose hydrolysate of cocksfoot grass using *Pichia stipitis* CBS 6054 after wet explosion pretreatment. The effect of wet explosion process parameters on the production of fermentation inhibitors such as acetic acid and furfural in the liquid fraction was evaluated.

2. MATERIAL AND METHODS

2.1. Wet explosion prepreatment

The Air-dried cocksfoot grass (*Dactylis glomerata*) was hammer milled to a particle size of 2-3 mm, and stored in plastic bags at room temperature prior to pretreatment. A portion of the raw material was ground in a coffee grinder to pass a 1 mm screen and used for chemical composition analysis.

The wet explosion (WEx) pretreatment was performed batch-wise with the following conditions: 160 ^{o}C-190 ^{o}C adding (at) 87 psi oxygen pressure (and) or at 0.2% dilute sulfuric acid concentration for 15 min (Table 1), by suspending the raw cocksfoot grass in tap water to reach a dry matter concentration w/w of 25% in a 10 L high-pressure reactor constructed at the Center for Bioproducts and Bioenergy, Washington State University, USA (Rana et al., 2012). The reactor was equipped with a gas/liquid inlet for injection of dilute sulfuric acid or oxygen pressure, and a continuous stirrer (2000 rpm). The reactor

was heated by a water jacket connected to a heat exchanger controlled by an oil heater. The temperature and pressure inside the reactor were monitored by two temperature sensors and one pressure sensor both mounted in the headspace and in the bottom of the reactor. The acid concentration or oxygen pressure was added into the pretreatment reactor after the desired temperature was reached. After the treatment, the biomass was flashed into a 100 L flash tank connected to the reactor, resulting in a sudden drop in temperature and pressure.

The resulting slurry from the pretreatment was separated into liquid and solid fractions by vacuum filtration. The solid fraction was stored in a freezer (-16 ^{o}C) for further processing and the filtrated liquid fraction was stored under refrigeration (5 ^{o}C) and used for ethanol fermentation by *P. stipitis*.

Table 1. Process conditions used for WEx pretreatment of cocksfoot grass.

Treatment	Temp. (^{o}C)	T/R* (min)	Oxygen (psi)	Acid concen.** (%)
A	160	15	87	-
B	170	15	87	-
C	180	15	87	-
D	170	15	-	0.2
E	180	15	-	0.2
F	190	15	-	0.2

*Retention time. **Acid concentration.

2.2. Preparation of WEx hydrolysate and fermentation

The hemicellulose hydrolysates used for all the fermentations were the liquid fraction obtained after separating the pretreated samples after WEx pretreatment from the solids, and were directly fermented to ethanol without enzymatic hydrolysis and detoxification. Fermentation was performed under semi-aerobic conditions in sterile 250 mL Erlenmeyer baffled flasks without any nutrient supplementation, covered with an aerobic stopper, and incubated on a rotary shaker at 125 rpm and 30 ^{o}C for 96 h as reported by Agbogbo and Coward-Kelly, (2008). The pH of the hydrolysates was adjusted to 6.0 with 1 M phosphate buffer solution.

2.3. Microorganism and media

Pichia stipitis CBS 6054 (ATCC 58785) was conserved and maintained on 20% glycerol at 4 ^{o}C at the Center for Bioproducts and Bioenergy, Washington State University, USA. *P. stipitis* inoculum medium contained 20 g/L D-xylose, 20 g/L peptone and 10 g/L yeast extract and was prepared aseptically in 250-mL shaking flask as previously described by Agbogbo and Wenger, (2007) with 100 mL medium and incubated on rotary shaker at 30 ^{o}C and 170 rpm for 24 h. All the media were sterilized by autoclaving at 121 ^{o}C for 30 min. The cells were harvested by centrifugation, and the pellet was collected for the hydrolysate fermentation to a final optical density (OD) of 1.0 measured at $OD_{600\ nm}$ corresponding to a cell concentration of approximately 1.7 g/L.

2.4. Analytical methods

The fermentation was performed in duplicates and monitored by withdrawing 2 mL of samples for analyses. The initial chemical composition of the raw material was determined according to the procedure developed by the National Energy Laboratory (Sluiter et al., 2008), and the dry matter content (DM), volatile solid contents (VS), and ash were determined according to the procedure described by the American Public Health Association (APHA, 1992). The concentration of sugars, acetic acid and ethanol were determined by high performance liquid chromatography (HPLC) refractive index (RI) equipped with an Aminex HPX-87P column (Bio-Rad Laboratories, CA, USA) at 83 ^{o}C with deionized water (Thermo Scientific, Barnstead Nanopure, IA, USA) as an eluent with a flow rate of 1.0 mL/min. The optical density (OD) of the yeast cell was measured spectrophotometrically at 600nm. The ethanol yield (Y_{EtOH}) was calculated by dividing the total amount of ethanol produced by the initial dry weight of treated cocksfoot grass. The percent theoretical (stoichiometric) ethanol yield (%Y_{EtOH}) was calculated according to Eq. (1): where 0.51 are the theoretical ethanol yield (in grams) generated per 1 g of sugar, Hatzis et al. (1996). This yield is always less than 100% as part of the sugars is converted to cell mass and by-products by the organisms.

$$Y_{EtOH}(\%) = \frac{Y_{EtOH}}{0.51} \cdot 100 \quad (1)$$

3. RESULTS AND DISCUSSION

3.1. Composition of WEx hydrolysates

The main chemical composition of raw material was (g/100 g DM): cellulose, 35.73; hemicelluloses, 23.71; and lignin, 18.74. The hydrolysates containing monomeric sugars and fermentative inhibitors used for the fermentations were prepared from the WEx liquid fractions and their compositions are depicted in Table 2.

Table 2. Composition of the WEx hemicellulose hydrolysates from wet exploded cocksfoot grass.

Compounds (g/L)	WEx process conditions					
	A	*B*	*C*	*D*	*E*	*F*
Hexose sugars	1.83 (0.01)	2.07 (0.04)	1.03 (0.03)	0.78 (0.02)	0.79 (0.01)	2.13 (0.03)
Pentose sugars	35.16 (0.07)	25.86 (0.04)	14.59 (0.05)	27.33 (0.16)	22.96 (0.17)	13.93 (0.13)
Furfural	0.44 (0.02)	0.50 (0.14)	2.90 (0.00)	0.17 (0.12)	0.51 (0.03)	1.00 (0.06)
Hydroxymethyfurfural	0.19 (0.00)	0.21 (0.08)	0.58 (0.15)	0.02 (0.00)	0.09 (0.05)	0.38 (0.11)
Acetic acid	1.72 (0.11)	2.13 (0.04)	5.21 (0.06)	1.32 (0.07)	2.04 (0.13)	3.06 (0.03)

Average of duplicates. Standard deviation shown in parentheses.

3.2. Fermentation of WEx liquid hydrolysates

The wet explosion liquid hydrolysates or fractions obtained from all the pretreatment conditions were fermented to ethanol by *Pichia stipitis* CBS 6054. Figure 1A and B shows the changes in ethanol and sugar concentrations among the WEx pretreatment conditions. Based on previous studies on hemicellulose hydrolysate fermentation by the yeast *Pichia stipitis* (Agbogbo and Coward-Kelly, 2008; Parekh et al., 1988), the aeration rate was kept

constant at 125 rpm throughout the fermentation, since oxygen is one of the crucial parameters for yeast *P. stipitis* during ethanol fermentation. Oxygen plays an important role in cell growth and generation of energy for xylose transport in *P. stipitis* (Agbogbo and Coward-Kelly, 2008). However, some studies on liquid hydrolysate fermentation by *P. stipitis* shows that genetically modified *P. stipitis* produces ethanol under anaerobic condition (Shi and Jeffries, 1998; Delgenes et al., 1986), but microaerobic conditions are optimal for ethanol production (Agbogbo and Coward-Kelly, 2008).

A rapid consumption of sugars was observed in most of the WEx conditions within the 24 h fermentation time. It is noteworthy that the available glucose in the fermentation broth was first consumed by *P. stipitis* before it started to utilize xylose and its complete uptake occurred in 96 h. The amount of ethanol produced steadily increased within 48 h fermentation time and leveled out after 72 h (Fig. 1A). A lag phase was not observed during the course of fermentation in most of the pretreatment conditions (Fig. 1B), except conditions (*C* and *F*) where metabolic activities was not detected due to high concentrations of fermentation inhibitors especially high contents of acetic acid associated with the above-mentioned conditions. The highest ethanol concentration obtained at the end of the fermentation (17.98 g/L) was achieved for the lower pretreatment severity, *A* (160 ^{o}C, 15 min, 87 psi oxygen), and it was in accordance with the utilization of sugars which amount to ethanol yield of 157.5 mL/kg DM, corresponding to 92% of theoretical maximum value (Table 3). This is comparable to ethanol yield of 85-90% of the theoretical maximum found for *Pichia stipitis* CSIR-Y633 fermenting xylose sugar (du Preez et al., 1986).

For the pretreatment conditions (*B* and *D*), the ethanol concentration was around 12 g/L, which is not comparable to the ethanol concentration found under condition *A*, but higher than the concentration achieved for condition *E*, which gave only approximately 10 g/L. This shows that the hemicellulose sugars under pretreatment condition *E* (170^{o}C, 15 min, 0.2% sulfuric acid) has to large extend been degraded to other products other than sugars, like furfural during the WEx pretreatment. However, the sugars found under the above-mentioned condition was able to ferment to ethanol, showing that the concentrations of inhibitors under this condition was not a limiting factor for the yeast *P. stipitis*, unlike conditions *C* and *F* (180 ^{o}C, 15 min, 87 psi oxygen and 190 ^{o}C, 15 min, 0.2% sulfuric acid)

where the yeast *P. stipitis* could not assimilate the sugars probable due to high content of inhibitors.

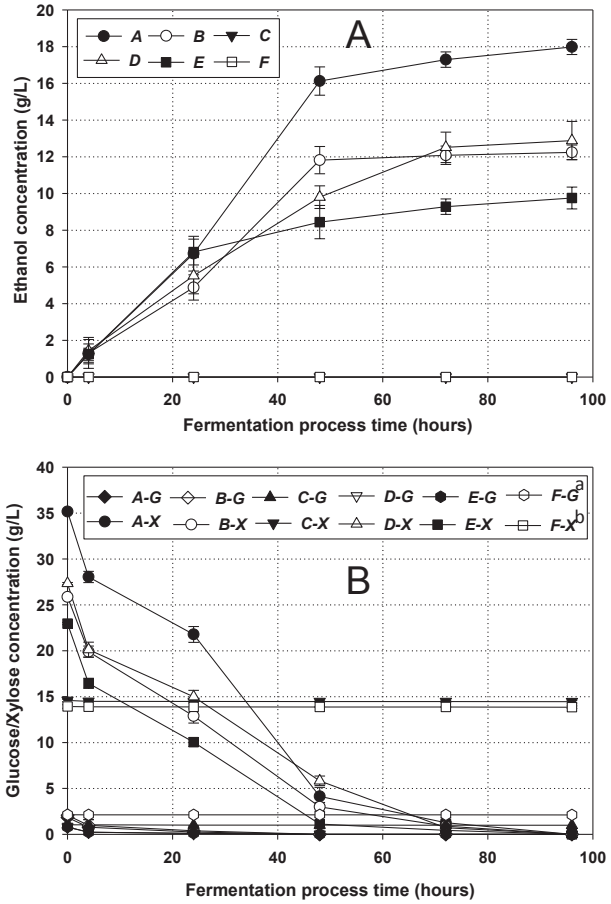

Fig. 1A. Time course of ethanol production from hemicellulose hydrolysates by *P. stipitis* CBS 6054 over 96h, 125 rpm at 30 ^{o}C and pH 6.0. (**B**) Time course of glucose and xylose consumption during ethanol fermentation over 96h. Values are means of duplicate experiments. [a]Glucose concentrations among the WEx pretreatment conditions. [b]Xylose concentrations among the WEx pretreatment conditions.

Pretreatment conditions *B* and *D* (170 ^{o}C, 15 min, 87 psi oxygen and 170 ^{o}C, 15 min, 0.2% sulfuric acid), shows a similar ethanol yield, but, was slightly higher in pretreatment condition *D* (Table 3), around 10% higher. The only difference in the above-mentioned conditions was the addition of pure oxygen and sulfuric acid. This is in agreement that pretreatment with addition of dilute acid at a moderate temperature can release up to 100% fermentable hemicellulose sugars and that a balance between solubilization and degradation of hemicellulose sugars is a mechanism in pretreatment with addition of both oxygen and sulfuric acid (Taherzadeh and Karimi, 2008). The above-mentioned WEx pretreatment conditions achieved ethanol yield of 112.3 and 123.7 mL/kg-DM, which corresponds to 65.8% and 72.4% of theoretical, respectively, (Table 3). In comparison, Zhong et al. (2009) reported ethanol yield of 72 and 68% of theoretical maximum, respectively, with *Pichia stipitis* FPL-061 and DX-26 fermenting AFEX-treated rice straw hydrolysates.

Table 3. Summary of fermentation results among the WEx conditions.

Treatment	Final ethanol concentration (g/L)	Ethanol yield (mL/kg-DM)	% of theoretical yield	Final pH
A	17.98 (0.02)	157.50 (0.05)	92.22	6.98 (0.02)
B	12.24 (0.04)	112.30 (0.03)	65.78	6.94 (0.05)
C	0.00 (0.00)	0.00 (0.00)	0.00	6.23 (0.03)
D	12.88 (0.04)	123.70 (0.06)	72.42	7.02 (0.04)
E	9.75 (0.01)	88.50 (0.02)	51.85	6.86 (0.08)
F	0.00 (0.00)	0.00 (0.00)	0.00	6.21 (0.07)

Standard deviation shown in parentheses. Fermentations were performed at 30^{o}C in a shaker incubator at 125 rpm over 96h.

The fermentability of WEx hydrolysates under pretreatment conditions *C* and *F* (180 ^{o}C, 15 min, 87 psi oxygen and 190 ^{o}C, 15 min, 0.2% sulfuric acid) was fully inhibited, because they contain high concentration of fermentation inhibitors. This demonstrates that lower pretreatment severity is more advantageous for maximizing the production of fermentable hemicellulose sugars thereby reducing the production of inhibitory compounds during

pretreatment. The above-mentioned conditions were the most severe pretreatment conditions tested in this study for WEx pretreatment with addition of oxygen or dilute sulfuric acid.

3.3. Effect of fermentative inhibitors

The inhibitory effects observed on the fermentation of WEx hydrolysates under pretreatment conditions (*C* and *F*) could be attributed to the presence of furfural at high concentration of about 2 g/L, but the complete inhibition of the fermentation could further be due to the higher concentrations of acetic acid (5.2 and 3.1 g/L, respectively) in the above-mentioned conditions (Table 2). It has been reported elsewhere in the literature (Roberto et al., 1991) that furfural concentration should be at a level of 1.0 g/L in order to present problems for yeast. The formation of acetic acid was more pronounced in the pretreatment condition with high temperature and addition of oxygen pressure. Palmqvist, (2000) and his co-worker reported in their recent review paper that microorganisms can up to a certain limit survive the stress of these compounds, but cell death would occur if the stress exceeds the limit that cell can bear. The effects of these fermentation inhibitors on ethanol fermentation by *P. stipitis* has been demonstrated in the literature, Bellido et al. (2011) found that ethanol yield from hemicellulose hydrolysates decreased with increasing acetic acid concentrations and uptake of xylose was more affected than glucose. This paper further mentioned that cell growth and ethanol yield was considerably affected at 2.5 g/L of acetic acid in synthetic media and complete inhibition of growth and ethanol production occurred at 3.5 g/L. Progressively, HMF and furfural caused delay of sugar consumption, but was eventually assimilated by *P. stipitis* below 2 g/L where inhibition was less profound than with acetic acid. Scordia et al. (2010) further reported that fermentation of hemicellulose liquid hydrolysates by *P. stipitis* is mainly inhibited by acetic acid and to lesser extent by the presence of furfural.

However, the liquid hydrolysates originating from any pretreatment of lignocellulosic biomass can be detoxified by removal of inhibitory compounds in order to adapt the yeast to utilize the available sugars to ethanol. Overliming and neutralization are some of the proposed methods to carryout hemicellulose hydrolysate detoxification (Cantarella et al.,

2004; Chandel et al., 2007). Performing hemicellulose hydrolysate detoxification is often energy demanding and can elevate the process cost of the ethanol production of hemicellulose sugars. In order to make lignocellulosic ethanol production more economically feasible, the hydrolysates arising from the separated liquid fractions after pretreatment should be able to ferment to ethanol without the need for further detoxification. Therefore, the hemicellulose hydrolysates obtained after the WEx pretreatment was not detoxified.

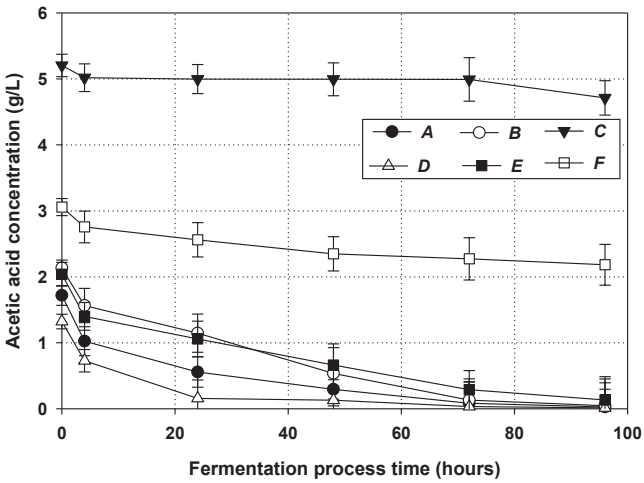

Fig. 2. Time course of acetic acid concentrations in the hemicellulose liquid hydrolysates during ethanol fermentation over 96h using *P. stipitis* CBS 6054.

Based on previous experiments with *P. stipitis* fermentation of hemicellulose hydrolysate (Ferrari et al., 1992), the initial pH in the fermentation broth for all the WEx pretreatment conditions were maintained at pH 6.0. At the end of the fermentation, an increase in pH was observed in most of the pretreatment conditions which can be attributed to the consumption of acetic acid by *P. stipitis* (Fig. 2). The acetic acid concentrations in most of the fermented WEx hydrolysates range from 1.32-2.13 g/L, but at the end of the fermentation, only about 0.1 g/L of acetic acid was found among the fermented WEx

hydrolysates. Table 3 shows the final pH range at the end of the fermentation among the pretreatment conditions. A pH range of approximately 7.0 was observed in most the pretreatment conditions, while the acetic acid was significantly consumed, however, the end products generated by *P. stipitis* from the acetic acid consumption was not determined. This is in accordance with the previous investigations on hemicellulose hydrolysate fermentation by *P. stipitis* where the increase in pH was attributed to acetic acid consumption (Agbogbo and Wenger, 2007; Scordia et al., 2010; Palmqvist and Hahn-Hägerdal, 2000).

4. CONCLUSIONS

This study has demonstrated that wet explosion (WEx) pretreatment with additives (dilute sulfuric acid or oxygen) facilitates the production of fermentable hemicellulose sugars that was optimally fermented to ethanol by *Pichia stipitis* CBS 6054 without further detoxification or use of costly enzyme mixtures. It further shows that lower pretreatment severity is an ideal combination of WEx pretreatment parameters for achieving higher ethanol yields from hemicellulose sugars, and at the time, reduces the formation of fermentation inhibitory compounds. This is evident as the highest ethanol yield of 158 mL/kg DM (92.2% of theoretical) was found under the lower pretreatment severity *A* (160 ^{o}C, 15 min, 87 psi oxygen). WEx hydrolysates obtained under higher pretreatment severity could, however, not be fermented to ethanol as it contains higher concentrations of inhibitory compounds.

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REFERENCES

Agbogbo, F. K., Wenger, K. S., 2007. Production of ethanol from corn stover hemicellulose hydrolyzate using *Pichia stipitis*. Ind. Microbiol. Biotechnol. 34, 723-727.

Agbogbo, F. K., Coward-Kelly, G., 2008. Cellulosic ethanol production using the naturally occurring xylose-fermenting yeast, *Pichia stipitis*. Biotechnol. Lett. 30, 1515-1524.

American Public Health Association (APHA), American Water Works Association (AWWA), and Water Environment Federation (WAE), 1992. Standard Methods for the Examination of Water and Waste Water. (Ed. Greenberg, A. E.., Clesceri, L. S., Trussel, R. R.), 18th edition.

Bellido, C., Bolado, S., Coca, M., Lucas, S., Gonzalez-Benito, G., Garcia-Cubero, M. T., 2011. Effect of inhibitors formed during wheat straw pretreatment on ethanol fermentation by *Pichia stipitis*. Bioresource Technology 102, 10868-10874.

Cantarella, M., Cantarella, L., Gallifuoco, A., Spera, A., and Alfani, F., 2004. Comparison of different detoxification methods for steam-exploded poplar wood as a substrate for the bioproduction of ethanol in SHF and SSF. Process Biochemistry 39, 1533-1542.

Chandel, A. K., Kapoor, R. K., Singh, A., Kuhad, R. C., 2007. Detoxification of sugarcane bagasse hydrolysate improves ethanol production by *Candida shehatae* NCIM 3501. Bioresource Technology 98, 1947-1950.

Chen, H., Qiu, W., 2010. Key technologies for bioethanol production from lignocellulose. Biotechnol. Advances28, 556-562.

Delgenes J. P., Moletta, R., Navarro, J. M., 1986. The effect of aeration on D-xylose fermentation by *P. tannophilus*, *P. stipitis*, *K. marxianus* and *C. shehatae*. Biotechnol. Lett. 8, 897-900.

Du Preez, J. C., Bosch, M., Prior, B. A., 1986. Xylose fermentation by *Candida shehatae* and *Pichia stipitis*: effects of pH, temperature and substrate concentration. Enzyme and Microb. Technol. 8, 360-364.

Ferrari, M. D., Neirotti, E., Albornoz, C., and Saucedo, E., 1992. Ethanol production from eucalyptus wood hemicellulose hydrolysate by Pichia stipitis. Biotechnol. and Bioengine. 40, 753-759.

Galbe, M., Zacchi, G., 2002. A review of the production of ethanol from softwood. Appl. Microbiol. Biotechnol. 59, 618-628.

Hatzis, C., Riley, C., Philippidis, G. P., 1996. Detailed material balance and ethanol yield calculations for biomass-to-ethanol conversion process. Appl. Biochem. Biotechnol. 96, 0273-2289.

Hendriks, A. T. W. M., Zeeman, G., 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresource Technology 100, 10-18.

Jeffries T. W., 2006. Engineering yeasts for xylose metabolism. Curr. Opin. Biotechnol. 17, 320-326.

Kristensen, J. B., Thygesen, L. G., Felby, C., Jørgensen, H., and Elder, T., 2008.Cell-wall structure changes in wheat straw pretreated for bioethanol production. Biotechnol. for Biofuels 1, 1-9.

Moniruzzaman, M., 1995. Alcohol fermentation of enzymatic hyrolysate of exploded rice straw by *Pichia stipitis*. World Jour. of Microbiol. Biotechnol. 11, 646-648.

Palmqvist, E., Hahn-Hägerdal, B., 2000. Fermentation of lignocellulosic hydrolysates II: inhibitors and mechanisms of inhibition. Bioresource Technology 74, 25-33.

Parekh, S. R., Parekh, R. S., Wayman, M., 1988. Fermentation of xylose and cellobiose by *Pichia stipitis* and *Brettanomyces clausenii*. Appl. Biochem. Biotechnol. 18, 325-338.

Petersson, A., Thomsen, M. H., Hauggaard-Nielsen, H., Thomsen, A. B., 2007. Potential bioethanol and biogas production using lignocellulosic biomass from winter rye, oilseed rape and faba bean. Biomass and Bioenergy 31, 812-819.

Rana, D., Rana, V., Ahring, B. K., 2012. Producing high sugar concentrations from loblolly pine using wet explosion pretreatment.Bioresource Technology 121, 61-67.

Roberto, I. C., Lacis, L. S., Barbosa, M. F. S., and de Mancilha, I. M., 1991. Utilization of sugarcane bagasse hemicellulosic hydrolysate by *Pichia stipitis* for the production of ethanol. Process Biochem. 26, 15-21.

Sassner, P., Mårtensson, C. G., Galbe, M., Zacchi, G., 2008. Steam pretreatment of H_2SO_4–impregnated Salix for the production of bioethanol. Bioresource Technology 99, 137-145.

Scordia, D., Cosentino, S. L., Jeffries, T. W., 2010. Second generation bioethanol production from *Saccharum spontaneum* L. ssp. *aegyptiacum* (Wild.) Hack. Bioresource Technology 101, 5358-5365.

Shi, X-Q., Jeffries, T. W., 1998. Anaerobic growth and improved fermentation of *Pichia stipitis* bearing a *URA1* gene from *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 50, 339-345.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., and Crocker, D., 2008. Determination of structural Carbohydrates and Lignin in Biomass. Laboratory Analytical Procedure, Technical Report NREL/TP-510-42618.

Taherzadeh, M. J., Karimi, K., 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review. Int. J. Sci.9, 1621-1651.

Taniguchi, M., Tohma, T., Itaya, T., and Fujii, M., 1997. Ethanol production from a mixture of glucose and xylose by co-culture of *Pichia stipitis* and a respiratory-deficient mutant of *Saccharomyces cerevisiae*. Jour. Ferment. Bioeng. 83, 364-370.

Zhong, C., Lau, M. W., Balan, V., Dale, B. E., Yuan, Y. J., 2009. Optimization of enzymatic hydrolysis and ethanol fermentation from AFEX-treated rice straw. Appl. Microbiol. Biotechnol. 84, 667-676.

Zhu, J. J., Yong, Q., Xu, Y., Chen, S. X., Yu, S. Y., 2009. Adaptation fermentation of Pichia stipitis and combination detoxification on steam exploded lignocellulosic prehydrolyzate. Nat. Sci. 1, 47-54.

Concluding remarks and future research

Stephen I. Njoku

CONCLUDING REMARKS AND FUTURE RESEARCH

The choice of a suitable pretreatment method and the adjustment of the pretreatment parameters are crucial for the efficiency of the subsequent conversion of any biomass in a biorefinery concept. The efficiency of the wet explosion pretreatment of different types of lignocellulosic biomass was successfully evaluated in terms of higher fermentable sugars production, ethanol conversion, and formation of degradation products as presented in the four research papers associated with the present PhD thesis.

High recovery of carbohydrates was achieved after the wet explosion pretreatment with addition of dilute sulfuric acid or oxygen pressure at 25% dry matter concentration. High sugar yields were obtained after enzymatic convertibility of the cellulose fractions up to 98% of theoretical maximum yield. Higher release of hemicellulose sugars in the liquid fractions was achieved with low formation of degradation products following pretreatment with oxygen at lower pretreatment temperature. Subsequently high ethanol yields were achieved with cellulose and hemicellulose conversion by *Saccharomyces cerevisiae* and *Pichia stipitis*, respectively, enabling economically viable bioethanol production in industrial scale. These findings clearly revealed that the adjustment of pretreatment parameters is dependent on the choice of end-products. High pretreatment severity – high production of hexose sugars and high formation of degradation products, and low pretreatment severity – high production of pentose sugars, lower production of hexose sugars and lower formation of degradation products. Therefore, pretreatment process parameters should be tailored to the specific biomass compositional structures and with a view to all the potential sugars and by-products which are aimed at.

This present study has demonstrated that wet explosion is an effective pretreatment method because it is flexible in feedstock handling and can operate with or without addition of chemicals. Its mode-of-action is divers because it consolidated the mechanisms of dilute acid, steam-explosion, and wet oxidation pretreatment. On the other hand, cocksfoot grass is a favorable feedstock for a cellulosic bioethanol production because of its high sugar content and is proven to be suitable for the BornBioFuel demonstration plant as tested in this present study.

Although the work presented in this PhD thesis addresses some features of biomass pretreatment and its conversion to ethanol, there is still the need for further investigation and validation of different process steps for the production of cellulosic bioproducts. In addition, the integrated production of biofuels together with other valuable coproducts will significantly enhance the cellulosic biofuels economics thereby reducing the technical and market risk in the context of biorefinery systems. The present process configurations should be further investigated on how to integrate the resulting materials after pretreatment into one single process line. The purpose would be to examine ways of optimum conversion of both hexose and pentose sugars in a continuous process configuration to reach a high ethanol concentration enabling economically feasible process.