Ethanol production from rape straw: Part of an oilseed rape biorefinery

Arvaniti, Efthalia

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Author: Efthalia Arvaniti

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Information Service Department

National Laboratory for Sustainable Energy

Danish Technical University

Postboks 49

4000 Roskilde

Danmark

Telefon 46774005

bibl@risoe.dtu.dk

Fax 46774013

www.risoe.dtu.dk

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PhD thesis

Efthalia Arvaniti

Submitted: December 2010

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"The whole is more than the sum of its parts"

Aristotle in Metaphysics (1045a10)

Preface

The present PhD project was carried out at the Bioenergy and Biorefinery program, Biosystems Division, National Laboratory of Sustainable Energy – Technical University of Denmark (Risø DTU), in the period September 15th, 2007 to December 4th, 2010. Principal supervisors were Senior Scientist Anne Belinda Thomsen (15/9/2007 - 31/08/2010) and Head of Program Jens Ejbye Schmidt (1/09/2010 - 14/12/2010. Co-supervisors were Senior Scientist Mette Hedegaard Thomsen (15/09/2007 – 31/01/2008), Jens Ejbye Schmidt (01/09/2008 – 30/09/2010) and PostDoc Zsófia Kádár (01/09/2010 – 31/12/2010). The project involved 1.5 months of research carried out during my external stay at Budapest University of Technology and Economics, Department of Applied Biotechnology and Food Science under the supervision of Professor Kati Réczey.

This work was part of the project "Biorefinery for sustainable reliable and economical fuel production from energy crops, Bio-REF", and it was mainly funded by Danish Strategic research council EnMi Project no. 09-061390. A short mission of 1.5 months was funded by COST FP0602 through STSM grant.

The aim of the study was to 1) present an oilseed rape whole crop biorefinery based on literature; 2) establish the best available experimental conditions for production of cellulosic ethanol from rape straw comprising the processes of thermo-chemical pretreatment, enzymatic hydrolysis, and C6 fermentation, and 3) couple cellulosic ethanol production to production of cellulolytic enzymes that are needed for cellulosic ethanol production, in a rape straw biorefinery.

In **Chapter 1**, the problem context and background theory for biorefineries is presented. Finally, latest developments of scaled up biorefineries in Europe are summarized. In **Chapter 2**, a scenario about upgrading and expanding a typical rapeseed biodiesel plant of Europe to an oilseed rape whole-crop biorefinery by 2020 is envisioned and discussed. The description and discussion of this biorefinery is based partly on literature review, and partly on own experimental data. Optimization of pretreatment of rape straw and production of cellulosic ethanol and cellulolytic enzymes from pretreated rape straw has been investigated separately by experiments in laboratorium. Experimental findings are cited directly with the papers that are added as **Paper I to III**. **Chapter 3** gives the overall conclusions from this thesis from both literature study and laboratory findings. At the end, **Chapter 4** gives a future outlook for further research on the studied topic.

Publication list

Paper I

Arvaniti E, AB Bjerre, JE Schmidt Optimization of wet oxidation pre-treatment of rape straw for releasing sugars for ethanol production. Biomass and Bioenergy, 2012 *In press* , DOI: 10.1016/j.biombioe.2011.12.040 Received 3 October 2010. Revised 13 November 2011. Accepted 21 December 2011.

Paper II

Arvaniti E, Z Benko, K Reczey, AB Bjerre, JE Schmidt. Production of cellulolytic enzymes using pre-treated rape straw as carbon source, Industrial Crops and Products, submitted

Paper III

Arvaniti E, A Thygesen, Z Kádár, AB Bjerre, JE Schmidt. Assessing best Simultaneous Saccharification and Fermentation conditions for ethanol production from pre-treated rape straw, Biomass and Bioenergy, 2010 submitted

Publications that were produced, but were not related to this thesis:

Conference proceedings

Thomsen AB, **E Arvaniti**, J Xu, P Oleskowicz-Popiel, T Fernqvist, N Schultz Jensen, Zs Kádár, M Jensen, ST Thomsen, A Thygesen, Pretreatment technologies for production of 2G bioethanol from agricultural waste and crops, 11th European Workshop on Lignocellulosics and Pulp, 16-19 August 2010, Hamburg Germany,

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Former and present colleges at the Biosystems Division, e.g. Zsuzsa, Xiaoru, Claes, Tinghong, two Henrik's, Sune, Morten, Mads, Hanne, Nadja, Stefan, Jian, Guantao, David, Anders, Simon, Wolfgang, Marcel, Erik, and Ulla are thanked for the amazing working atmosphere they produced at NRG. You have been all like a family for me. Many thanks to Piotr for being a very cool and inspiring office-mate for three years. Thousand thanks to the technicians of our group: Tomas, Ingelis and Annette for their excellent systematic work, light speed, and kind assistance. They are the pillars of the program.

Also, I would like to thank Adam Dabrowski who allowed me use his painting "Rapeseed field" in the front-page.

I would like to thank all my friends in Greece, in Denmark, and all over the world for being such bright stars in my life, showing me the way. Special cordial regards are given to my lifetime sister-friend Yota, and adult sister-friends Anna and Loukia for the evergreen heartful long distance relationships all these years.

Also, I want to thank my tango and theater friends and teachers, that although they didn't directly contribute to this thesis, they definitely blended me with beautiful and cheerful moments.

Finally, I would like to thank my parents, grandparents, and my brother for always being there (in Greece) for me. Αγαγημένοι μου μαμά και μπαμπά και αδερφούλη, ευχαριστώ πολύ που είστε τόσο υπέροχοι και λαμπεροί. Χωρίς την εύφορη συμπαράστασή σας, την παραδειγματική καλοσύνη σας και το αστείρευτο χιούμορ σας, αυτή η διδακτορική διατριβή δε θα είχε γραφτεί. Το βιβλίο είναι αφιερωμένο σε εσάς παμψηφεί!

And last but not least, I want to thank my darling Bjørn for his superb and catalytic presence in my life.

Contents

Summary

Presently, due to depletion of fossil fuels and concerns over climate change, there is a wish to substitute fuels and materials based on fossil deposits with renewable and biobased ones. This trend combined to the plethora of goods, like energy, chemicals and materials that are produced from fossil-sources, creates a huge demand for development of agricultural and biomass processing systems. In addition, agriculture has to continue to feed a growing population. Efficient exploitation of primary crops, crop residues and wastes for energy and products will determine how realistic a biobased economy will be. This will be accomplished with the development of biorefineries.

Rapeseed biodiesel is an established product in Europe with an annual production of 9 million tons in 2009. However, the economic potential of residues from this industry (rapeseed cake, rape straw, crude glycerol), which represent 82 % of the original plant material, have not been fully exploited. Currently, only low-grade applications are found in the market. As a consequence, it was deemed of interest to develop a technological platform aiming to convert rapeseed biodiesel residues into value-added products. Specifically, a plan was made to expand and upgrade an existing biodiesel plant to become an oilseed rape biorefinery by 2020. Within this time frame the choice of products was based on a technological feasibility study. Priority was given to Low-Value-High-Volume products which could be marketed easily. Apart from rapeseed biodiesel the products selected were: ethanol, biogas, products for combustion, enzymes, chemical building blocks, and finally animal fodder of superior quality. The production lines were analyzed and prospects for 2020 were projected. Particular attention was given to two products, i.e. ethanol produced from cellulose and cellulolytic enzymes produced from rape straw.

Production of ethanol from the cellulose of rape straw was optimized with respect to all production steps, i.e. for thermo-chemical pretreatment, enzyme hydrolysis, and fermentation of C6 sugars. Thermochemical pretreatment was carried out using the “Wet Oxidation” technique (WO) under different conditions, i.e. temperature, reaction time, oxygen pressure. Also, configurations like recycling liquid (filtrate) in WO, presoaking of rape straw in water or recycled filtrate before WO, skip washing pretreated solids (filter cake) after WO, or use of whole slurry for ethanol production were tested. The following indicators are used to assess the suitability of the process: (1) Ethanol yield, (2) amount of water used, (3) recovery of cellulose, and finally (4) recovery of hemicellulose and lignin. The results have shown that WO treatment at 2-3 minutes achieved highest ethanol yields, and highest sugar and lignin recoveries. Also, recycling liquid in WO and increasing oxygen gas pressure did not improve ethanol production, but increased glucose yields and reduced recovery of sugars and lignin. Finally,

use of filtrate could inhibit ethanol production, but also decreased lactic acid formation in SSF. The highest ethanol yield obtained was 67% after fermenting the whole slurry produced by WO at 205 °C for 3 min with 12 bar of oxygen gas pressure featured with presoaking in water. At these conditions after pre-treatment, cellulose and hemicellulose was recovered quantitatively together with 86% of the lignin.

Optimization of ethanol production from rape straw was then focused on identifying the best enzyme mixture for hydrolyzing cellulose and hemicellulose of pretreated rape straw by WO. Both commercial enzymes (Celluclast, Cellubrix), and produced enzymes were tested. Enzymes were produced with four fractions of WO pre-treated rape straw: (1) moist filter cake, (2) whole slurry, (3) partly detoxified whole slurry, and (4) partly detoxified filtrate were applied as carbon source for *Trichoderma reesei*. All enzymes are evaluated by the achieved glucose and xylose yields with WO pretreated rape straw. In addition, produced enzymes are evaluated by the achieved enzyme activity yields. For hydrolysis of cellulose, highest glucose yields were achieved with commercial enzymes (77-79%). For hydrolyzing hemicellulose, highest xylose yield was achieved by the commercial enzymes and by the enzymes produced with Solka floc (which was the reference carbon source) ranging 74-78%.

Among the fractions of pretreated rape straw, filter cake and whole slurry induced the highest FPA yields at 123 FPU/g monosaccharide equivalents (at 11 days). Glucose and xylose yields for enzymes produced with whole slurry were 71-72%, and 66-74% respectively. Detoxification of whole slurry did not improve enzyme production. The β-glycosidase activity was very low in all enzyme mixtures (commercial and produced). As for enzymes produced with whole slurry it was estimated that about 20% of pretreated rape straw filter cake needs to be allocated for enzyme production (carbon source), while the rest is available for bioethanol production.

Finally production of ethanol after applying established pretreatment WO and employing the most effective enzyme mixture was optimized in Simultaneous Saccharification and Fermentation. The evaluation was based on obtained ethanol yields. Three pure cultures of *S. cerevisiae* and a baker's yeast were tested on thermotollerance, low pH, and increased dry matter (DM). SSF of whole slurry with baker's yeast showed that adjustment of pH before inoculation was important for achieving high ethanol yields (68%). There were no significant difference in the ethanol yields at 32 °C (70-75%) and 37 °C (72-76%) for the three pure cultures. However, SuMo (isolated from baker yeast) and Turbo (isolated from a brewing yeast) achieved higher ethanol yields at 40 °C (64-66%), compared to ATCC 96581 (57%) after 115 hours of SSF. Increasing DM from 12.5% to 16% during SSF experiments with SuMo yeast at 37 °C for120 hours did not have any significant influence on the ethanol yield (75-76%), while further increase of the DM content to 18% resulted in a decrease of the ethanol yield to 68%. In SSF

experiments in a fermenter of 1 litre working volume with a DM of 12.5% and a fixed pH to 4.8 resulted in an ethanol yield of 63% compared to the shake flask reference. Moreover, lactic acid was formed as by-product (11 g/l).

Resume

På grund af udtømning af de fossile brændstofressourcer samt bekymringer omkring klimaændringer, vokser ønsket for at erstatte fossilt baserede varer med produkter der kan produceres baseret på biomasse og dermed på en vedvarende måde. Denne tendens, kombineret med den nuværende overflod af varer, såsom energi, kemikalier og materialer, der er fremstillet fra fossile kilder, skaber en enorm efterspørgsel efter udvikling af landbrugs- og biomassesystemer. Ud over dette, skal landbruget stadigt udfylde sin traditionelle rolle som leverandør af fødevarer og foder, til at understøtte en stadigt voksende befolkning. Effektiv og bæredygtig udnyttelse af kulstof og energi fra primære afgrøder, afgrøderester og affald vil tegne fremtiden for den bio-baserede økonomi. Dette kan ske via udviklingen af bioraffinaderier.

Den europæiske industrisektor for rapsbaseret biodiesel er blevet etableret med succes og havde i 2009 en årlig produktion på 9 mio. tons. Restprodukterne fra denne produktion (rapskage, rapshalm, rå glycerol), som i alt udgør 82 % af massen af rapsafgrøden, er dog ikke er blevet udnyttet til fulde, og de har i øjeblikket kun ringe værdi på markedet. Derfor er der i dette studie blevet opbygget et scenario med en kvalificeret udnyttelse af resterne fra rapsbaseret biodiesel, dels for at danne værdiforøgede produkter, dels for at kunne udvide og opgradere et eksisterende biodieselanlæg, samt med det mål at have et rapsbaseret bioraffinaderi på europæisk grund i 2020. Udvælgelsen af produkter blev baseret på en teknologisk forundersøgelse i henhold til den givne tidsramme, mens "Low-Value-High-Volume" produkter der er lette at afsætte, såsom energi- og foderprodukter, blev prioriteret. Ud over den rapsbaserede biodiesel var de udvalgte produkter, ethanol, biogas, enzymer, energi, kemiske byggesten og høj-kvalitets dyrefoder. Disse produktionslinjer blev analyseret og udsigterne for 2020 blev projiceret. Særlig fokus blev givet til to produkter, ethanol fra cellulose, og cellulolytiske enzymer fra rapshalm.

Produktionen *af cellulosebaseret ethanol* fra rapshalm blev optimeret i alle produktion trin, dvs. den termokemiske forbehandling, enzymhydrolysen, og gæringen af C6 sukre. Termo-kemisk forbehandling blev undersøgt med vådoxidation (WO) ved forskellige temperaturer, reaktionstider, og ilttryk, men også andre faktorer som recirkulering af den flydende fase (filtrat) i WO, iblødsætning af halm i varmt vand og i recirkuleret filtrat før WO, udladning af vask af det forbehandlede filterkage, såvel som brug af både flydende og faste fase (hele slurry) til ethanol produktion, blev undersøgt. Følgende indikatorer er brugt til at vurdere egnethed af processerne: (1) ethanoludbytte, (2) vandforbrug, (3) genfindelse af cellulose, og (4) genfindelse af hemicellulose og lignin. Resultaterne viste at WO-behandling med en varighed af 2-3 minutter opnåede de højeste ethanol udbytter og den højeste genfindelse af sukker

og lignin. Recirkulering af den flydende fase i WO og forhøjning af ilttryk forbedrede ikke ethanol produktionen, men forhøjede glucose udbyttet og reducerede genfindelsen af sukker og lignin. Bruget af filtratet hæmmede ethanolproduktionen og reducerede dannelse af mælkesyre i SSF. Det højeste ethanol udbytte var 67%, opnået efter fermentering af hele slurry produceret af WO ved 205° C i 3 minutter og 12 bar ilttyrk med forudgående iblødsætning i vand. Genfindelsen af cellulose og hemicellulose under disse betingelser var kvantitative, mens genfindelsen af lignin var 86 %.

Derefter fokuseredes optimeringen af ethanolproduktion fra WO-forbehandlet rapshalm på at identificere de bedste enzymblandinger for hydrolyse af cellulose og hemicellulose. Der blev tested både kommercielle enzymer (Celluclast, Cellubrix) og producerede enzymer. Enzymer blev produceret med fire fraktioner WO forbehandlede rapshalm: (1) fugtige filterkage, (2) filterkage og filtrat, (3) delvis afgiftet filterkage og filtrat, og (4) delvis afgiftet filtratet blev anvendt som kulstofkilde for Trichoderma reesei. Alle enzymer blev evalueret af de opnåede glucose og xylose udbytter fra WO-forbehandlet rapshalm. Derudover blev enzymerne evalueret af de opnåede enzymaktivitet-udbytter. For hydrolyse af cellulose, opnåede kommercielle enzymer det højeste glukoseudbytte (77-79 %). For hydrolyse af hemicellulose opnåede kommercielle enzymer og enzymer produceret af Solka floc (som var reference kulstofkilde) det højeste udbytte af xylose (74-78%).

Blandt de fraktioner af forbehandlet rapshalm, gave filterkage og hele slurry det højeste FPA udbytte på 123 FPU / g monosaccharide ækvivalenter (på 11 dage). Glucose og xylose udbytter for enzymer produceret med hele slurry var henholdsvis 71-72%, og 66-74%. Afgiftning af hele slurry førte ikke til bedre enzymproduktion. β-glycosidase aktiviteten var meget lav i alle enzymblandinger (kommercielle og producerede). Ligesom for enzymer fremstillet med hele slurry blev der anslået, at omkring 20% af de forbehandlet rapshalm filterkage skal tildeles for enzymproduktion (kulstofkilde), mens resten kan bruges til fremstilling af bioethanol.

Endelig blev ethanol fermentering optimeret i SSF med den valgte forbehandlingsmetode, og den bedste enzymblanding. Evalueringen er baseret på det opnåede ethanoludbytte. Tre rene kulturer af S. cerevisiae og en bagegær blev testet på thermo-tollerance, lav pH, og øget tørstof (DM). SSF af hele slurry med bagegær viste, at justering af pH før inokulering var vigtigt for at opnå et højt ethanol udbytte (68%). Der var ingen signifikant forskel i ethanol udbyttet ved 32 °C (70-75%) og ved 37 °C (72-76%) for de tre kulturer. Imidlertid opnåede Sumo (isoleret fra bager gær) og Turbo (isoleret fra en bryggegær) en højere ethanol udbytte ved 40 °C (64-66%), sammenlignet med ATCC 96.581 (57%) efter 115 timer i SSF. Øgningen af DM fra 12,5% til 16% i et SSF forsøg med Sumo gær ved 37 ° C for120 timer havde ikke væsentlig indflydelse på ethanol udbytter (75-76%), mens yderligere øgning af DM-

indholdet til 18% resulterede i et fald i ethanol udbyttet til 68%. SSF eksperimenter udført i en fermenteringstank på 1 liter aktiv volumen, med et DM på 12,5% og en fast pH på 4,8 resulterede i et ethanol udbytte på 63%, sammenlignet med udbyttet fra shake flask referencen. Desuden blev mælkesyre dannet som biprodukt (11 g / l).

1 BIOREFINERIES

1.1 Introduction

Fluctuating prices of fossil fuels [1], climate change concerns [4], and political drives [5] have increased the demand for renewable resources for production of energy and materials. The transportation sector is responsible for 60% of the oil consumption in the OECD countries [6]. The importance of fossil fuels is underscored by the fact that that they contribute 98% of the transport fuels (2010) [6], and 94% (2006) of organic chemicals and plastics [7]. An alternative solution is to use the chemical energy stored in biomass currently produced by the sunlight [3]. Many governments and organizations around the world have targeted substituting fossil fuels by bio-based ones. EU has set the target that biofuels should contribute 10 % to the transport sector by 2020 [8]. Equivalent targets for bio-based products are missing in EU policies, but US has adopted a target of 18% for 2020 and 25% for 2030 [9] (See Table 1).

Table 1 Share of bio-based fuels and products on EU and USA policies (adapted by [2,5,10]).

	Year	2010	2020	2030
US	Biofuels (%)	4	10	20
	Bioproducts (%)	12	18	25
EU	Biofuels (%)	5.75	10	-
	Bioproducts (%)	8 (2001)	-	-

A shift of production of fuels and chemicals from fossil-based to bio-based, faces two major challenges: (1) there is a definite amount of arable land available [11] to cover a growing market for food, feed, materials (fibers),

chemicals and energy; (2) when compared to the use of fossil energy, production of biobased fuels, chemicals, and materials there is not necessarily a benefit for the environment in terms of mitigating GHG emissions and/or reducing pollution of the environment. There have been many cases where petro-based products were less detrimental to the environment than bio-based ones [12]. Special attention needs to be given to both problems to secure a sustainable biobased economy for the future. Specifically, economic biomass production and processing is needed which allows the combined production of food, feed, energy, chemicals, and materials. These demands can be covered by whole-crop biorefinery concept.

1.2 From petro-refineries to biorefineries

Biorefineries, although invented parallel in time to petro-refineries about 150 years ago [9], are still in an early stage compared to mature petro-refineries. Nowadays, there are plenty of simple biorefineries like plants for dry-mill ethanol or soy-bean biodiesel, but in terms of efficiency and flexibility they are far behind the petro-refineries. A list of analogies between petro-refineries and biorefineries are listed in table 2. Although the general idea is the same, the starting materials and the production processes vary considerably. For instance, unlike petro-refineries, biorefineries prioritize environmental sustainability of production in LCA analysis

Biomass is overall more oxidized than the hydrocarbons present in the oil [13]. Therefore the chemistry of the petro-refinery and biorefinery processes can be very different. For instance, oxygen rich sugars are very good building blocks for bioproducts but less suitable for biofuels due to low heating values (LHV) [13]. During the conversion of carbohydrate to ethanol the energy content per carbon is increased through disposal of oxygen in form of CO_2 [13]. Moreover unlike oil, biomass feedstocks can vary in composition and quality from harvest to harvest. This variation has

been the strongest argument of chemical industries against switching their activities from fossil to bio-based resources [14].

Table 2 Analogies for petro-refineries and biorefineries

	Petro-refineries (adapted by [15])	Biorefineries
Primary resource composition and diversity	Standard quality with small variations per batch	Diverse quality and composition of biomass, ranging for species, breed, cropping conditions, year
Platforms	Highly reduced compounds: Hydrocarbons (C1-C50), like olefins, alkanes, cycloalkanes, aromatics	Both reduced and oxidized compounds: Sugars (C5/C6), oils, lignin, Biogas, Syngas, pyrolytic liquid, organic juice, hydrogen, electricity and heat[3]
Production process of platforms	Distillation	Pretreatment of biomass (physical, chemical, thermal, biological, combinations)[16,17]
Working temperatures of the refinery	Medium high temperatures	Diverse: biological processes need mild temperatures, otherwise high temperatures
Current drive	Economical (growth, job development); Societal (Cover market needs, reliability); Technological (Process refinement, well discovery and extraction); Political (Energy security)	Economical (green growth, job development especially in rural areas); Societal (Cover market needs, green ethical products); Environmental (Sustainability, Renewable energy, GHG abatement, new biodegradable bio-products, recycling/effective use of wastes); Technological (opportunity for innovation, education); Political (energy security of supply)
Processing capacity scale	Typically 200,000 bar/day; 10Mibar/y	Pilot/Demo scale
End-products	Myriads (Fuels, commodity chemicals, materials, heat, electricity, etc.)	Constantly developing new products and process routes (biofuels, bio-based chemicals, biomaterials, electricity, heat, etc. [9])
Process development	Little; The technology is mature; Catalytic activities will improve conservatively	Drastically; The technologies are taking off and projects are upscaled; Biocatalysts are excepted to improve in terms of efficiency, product yields, productivities, and purchasing costs
Product development	Top down (given the characteristics of oil, products and processes were developed)	Two ways: top-down and bottom-up: Bioproducts need to substitute petro-refinery products, but also new bio-products/markets will develop based on the properties of the biomasses

1.3 Classifications

According to IEA Bioenergy Task 42, “Biorefining is sustainable processing of biomass into a spectrum of marketable products and energy”. A biorefinery is a scheme to utilize all components and properties of biomass for product manufacturing and energy extraction with practically no waste.

A classification method for biomasses for biorefineries is given by Wellisch et al. 1) *Primary biomasses* are harvested directly from land or the water. 2) *Secondary biomasses* are processing residues of the food or feed industries, and finally 3) tertiary biomasses are post-consumer wastes[18]. Although secondary and tertiary biomasses are available at lower prices than primary biomasses and are suitable feedstocks for bulk chemicals and biofuels, Wellisch et al (2010) [18] underlines that other product-driven biorefineries might have specific needs on feedstock structure or chemistry that only biomasses can fulfill.

Classifications of biorefineries found in literature are numerous, and are based on the complexity of the design [9], the technological platforms involved (thermo-chemical, biological etc), or a principal characteristic of the biorefinery (green biorefinery, integrated biorefinery, lignocellulosic biorefinery, conventional etc. [19]). This plurality and incomprehensiveness in classification create a considerable confusion in communicating on biorefineries.

The most intuitive, but yet not comprehensive classification methods was developed by Kamm et al,2004 [2]. In this study, the biorefineries were classified based on their complexity of design (see Figure 1): Type I biorefineries have one starting material and one major product, and in addition, the production process is relatively simple. Examples of such biorefineries are existing rapeseed biodiesel plants, dry mill ethanol plants and or pulp/paper mills. In these projects, all effort is given to purify a single component of the feedstock, whereas the residues are complex and mixed (like the black liquor from pulp and paper industry). These residuals are marketed as low grade products with no or only limited further processing. Such biorefineries are all well established in full scale, and they are the precursors of phase II biorefineries. Phase II biorefineries utilize again one feedstock, but unlike phase I biorefineries, integrate a more complicated, often multi-branched, production process, with numerous products similar to a typical petrochemical refinery. Unlike phase I biorefineries, phase II biorefineries valorize each major component of feedstock by processing it into a product, and are more flexible in

production output. While phase I biorefineries, are abundant, phase II biorefineries are more rare. Examples are wet-corn mills, wet-wheat mills and industries with integrated anaerobic wastewater treatment combined with production of energy. Examples of phase I and phase II biorefineries will be analyzed in further detail in the next paragraphs. Finally, Phase III biorefineries are biorefineries that combine use of multiple substrates and products. A simple example of phase III biorefinery could be an anaerobic digestion plant that uses a multitude of secondary and tertiary sources of biomass for production of CH_4 and CO_2, and concentrated fertilizer (N, P). This example is similar to the example of phase II biorefinery but is referring to many and diverse sources of substrates. So far, no other phase III biorefineries have been developed.

A comprehensive classification method for biorefineries [19] was given by Cherubini et al in 2009 [3], listing in order of appearance the platforms involved, the final products formed in the biorefinery, the biomass resource(s) used, and finally the principal processes. For example, a typical rapeseed biodiesel plant, according to Cherubini et al (1999) [3] is, "one platform (oil) biorefinery for biodiesel, glycerol, and animal feed, from rapeseed". The platforms are the main intermediates (pillars) that link feedstock and final products (like oil, C6 sugars, C5 sugars, lignin, biogas, etc). Principal processes could be mechanical, chemical, biological or thermochemical. This classification enclosed all information required for a one-line biorefinery description. The Kamm classification is included since differentiation between phase I and II is depited in the amount of platforms used and products formed, while the amount of feedstocks utilized diffrentates the complexity of the biorefinery between phase II and III is. The weak point of this classification is that it does not include the scale of the biorefinery, which needs to be added as R&D/pilot/demo/full.

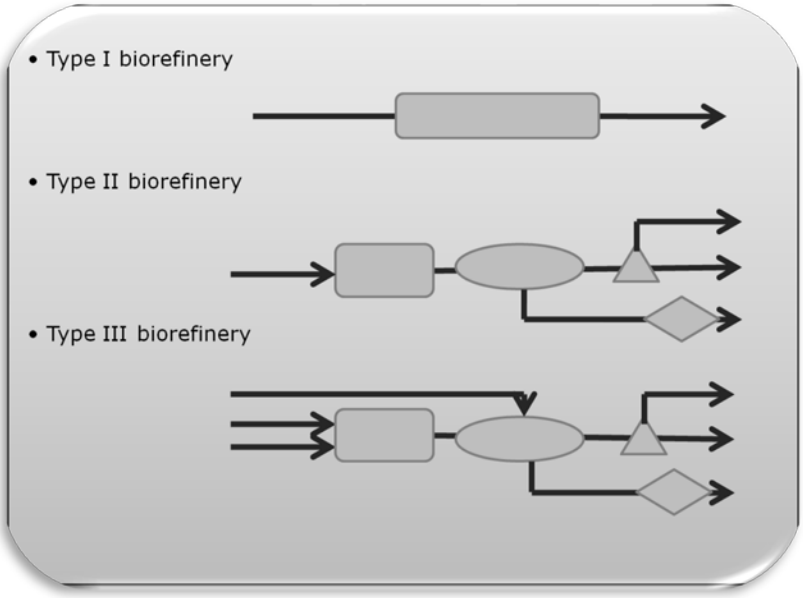

Figure 1 Classification of biorefineries based on the complexity of the design, based on Kamm et al. 2004 [2]

1.4 State of the art of simple biorefineries

The most successful and widely spread biorefineries worldwide are dedicated to production of 1G biofuels, i.e. bioethanol, biodiesel, derived from dedicated crops containing suchrose, starch, triglycerides [20]. In 2009 9 MiT of biodiesel were produced in EU, primarily from rapeseed, well below the 22 MIT production capacity [21]. Residues from rapeseed biodiesel industry are rapeseed cake, crude glycerol and the rape straw left in the field. In many cases, biodiesel plants purchase rapeseed oil (pressing is not including in process configuration). Crude glycerol and rapeseed cake are sold to the market for low grade applications, and wastewater is discharged to municipal water treatment. According to Cherubini this biorefinery this is *"One platform (oil) biorefinery for biodiesel, glycerol, and animal feed, from rapeseed".*

A second type of simple biorefinery that are mature worldwide is the dry grain mills, like Ensus in UK, with produces ethanol, Dry Digestible Solids with Solubles (DDGS) and CO_2, A typical dry wheat mill biorefinery yields 380 liters of ethanol, 350 kg of DDGS, and 350 kg CO_2 per ton wheat grain. DDGS contains the spent grains, and the stillage concentrate, and is sold for low-grade applications. Dry grain mills can be classified as: *"One platform (C6 sugars) biorefinery for ethanol, animal feed, and CO_2 from wheat".*

A third type of well-known simple biorefinery is the pulp and paper mills. Pulp and paper industries typically use wood that is treated chemically or mechanically to separate cellulose fibers for production of paper pulp. The residues of this process, i.e. hemicellulose and lignin, are concentrated and burned in boilers for energy recovery. The ashes collected in the oven are recycled back to the chemical pulping process. This is therefore, *"Two platforms (C6 sugars, electricity and heat) biorefinery for pulp and electricity and heat, from wood".*

1.5 State of the art of complex biorefineries

Complex biorefineries are few but they do exist. Such a plant can develop either, by expanding or modifying a simple biorefinery, or by designing and building it from scratch [18]. In many cases, the first option may keep down capital investments [22]. For example, since 2007 Neste in Finland has used rapeseed oil in an existing petro-refinery [23] for production of alkanes [24]: *"One platform (oil) biorefinery for alkanes, from rapeseed".*

Table 3 lists some of the current or planned complex biorefineries in Europe (at least demo scale). This list does not contain simple biorefineries like the ones mentioned before. In addition to scaled up projects, a few pilot-scale activities are selectively included.

Wet-grain mill biorefineries are among the most mature complex biorefineries worldwide. Compared with dry-wheat mills this type of biorefinery has a broader range of products, separating several

components out of the feedstock. For example, Archer Daniel Midland USA has 8 wet wheat mills in UK alone, producing starch, high-fructose wheat syrup, ethanol, germ seed oil, gluten, and meal. Such wet wheat mill is then *"three platform (C6 sugar, oil, organic juice) biorefinery for ethanol, several food ingredients, and feed from wheat".*

An example of expanded complex biorefineries from simple biorefineries is the integration of anaerobic wastewater treatment facilities to existing food related production industries [25]. In the last decades because anaerobic digestion technology has become so profitable and flexible on feedstock, it has become popular in coupling industrial scale biorefineries worldwide. Nowadays many industrial wastes (food and beverage, agricultural, chemical, pulp and paper etc.), are treated and produce biogas [26] This technology accoding to Cherubini and colleagures [3] is "one platform (biogas) biorefinery for biomethane and fertilizer from industrial wastes (variable)".

***Table 3** List of selected complex biorefineries in EU territory either built or planned. Simple biorefineries like rapeseed biodiesel, and dry-mill cereal ethanol are not included. The biorefineries are listed alphabetically, and according to Cherubini and colleagues classification method [3] where the biorefineries are characterized on the platforms involved, the products formed (energy, materials), the utilized biomass feedstosks, (primary, secondary, tertiary), and production scale.*

Biorefinery Project name, Country	Platform	Products		Feedstock		Scale	Reference
		Energy	**Materials**	**Primary**	**Secondary/tertiary**		
ADM, UK	C6 sugars, oil	Ethanol	Food ingredients (starch, sweeteners, protein), seed oil, feed	Wheat	-	Full (8x)	http://www.admmilling.co.uk
Algomed, DE	Oil	-	Fatty acids, lipids, carotenoids	Micro-algae	-	Demo	http://www.algomed.de
BioAmber, FR	C6/C5 sugars,	-	Succinic acid	C6/C5 sugar sources	Lignocellulose sugars, glycerol, CO_2	Full	http://www.bio-amber.com/img/pdf/jan20_2010.pdf
BornBiofuel2, DK	C6/C5 sugars, lignin, biogas	Ethanol, electricity, heat, methane	Hydrogen, salts	-	Wheat straw, garden waste, grasses	Demo (planned)	[27], http://www.biogasol.com
Bigadan, DK	C6/C5, organic juice, Biogas	Methane	-	Industrial waste, manure		Full (many)	http://bigadan.dk/eng
Bioliq, DE	Pyrolytic liquid	Customized fuels	Synthesis gas, chemicals	-	Straw, wood	Pillot (planned 2016)	http://www.bioliq.de/
Bioro, BE	Oil	Biodiesel	Crude glycerol	Oilseed rape	-	Full	http://www.bioro.be
BioTFuel, FR	Gasification,	FT fuel	-	Wood	-	Pilot, x2	
Biowert, DE	Organic juice	-	Fertilizer, biomaterials, animal feed	Grass	-	Pilot	http://www.biowert.de

Boregaard, NO	C6/C5 sugars, lignin	Ethanol	Lignosulfonate-based binding and dispersant agents, chemical grade cellulose, fine chemicals (pharma), food additives	Wood	-	Full	http://www.borregaard.com/
BornBiofuel2, DK	C6/C5 sugars, biogas, lignin	Ethanol, methane, hydrogen	Electricity and heat	Grasses	Garden waste, straw	Demo	http://biofuels.abc-energy.at/demoplants/img/files/related_publications/367/367_2009-03-09-120823_related_publications.pdf
British airways, UK	Syngas	Synthetic biofuels, electricity, heat	Construction material	-	MSW	Full (planned 2014)	http://news.bbc.co.uk/2/hi/8515620.stm
ChemPolis, FI	C6/C5 sugars, lignin	Ethanol, electricity and heat	Chemicals (Furfural, acetic acid, formic acid), paper fibers, potassium fertilizer	Bamboo	Straw, bagasse, corn stover	Demo	http://www.chempolis.com/
Chemrec, SE	Syngas	DME, methanol	-	-	Black liquor	Pilot (and Full scale planned)	http://www.chemrec.se
Choren, DE	Syngas	Synthetic biofuels, electricity and heat	-	Wood	Wood residues	Demo (2x)	http://www.choren.com
Chrisgas, SE	Syngas	-	Hydrogen-rich syngas	-	Wood chip and straw pellets	Pilot	http://www.chrisgas.com
Cristanol, FR	C6 sugars	Ethanol	Feed, polyphenols (planned)	Sugar beet, cereal	-	Full	http://www.cristal-union.fr

CyL Biomass Plant, ES	C6/C5 sugars	Ethanol	Feed	-	Wheat straw	Demo	http://www.abengoabioenergy.com
Daka, DK	Oils	Biodiesel, heating oil	Glycerol, Potassium sulfate	-	Animal fat	Full	http://www.dakabiodiesel.com
Domsjö, SE	C6/C5 sugars, lignin	Ethanol, heat	Cellulose fibers,lignin	Wood	-	Demo	http://www.domsjoe.com/
FibreEtOH, FI	C6/C5 sugars, lignin	Bioethanol, biogas, electricity and heat	-	-	Pulp and paper mill waste, waste log, waste fibre	Pilot, Demo (planned)	http://www.upm.com
Futerro, BE	C6 sugars	-	PLA	Sugar beat, sugar cane, corn	Forest and agricultural wastes (planned)	Pilot	http://www.futerro.com/
Futurol, FR	C6/C5 sugars, lignin	Bioethanol, electricity and heast	Hydrolytic enzymes	-	Lignocellulosic biomass, vinasses	Pilot	http://www.projet-futurol.com/index-uk.php
Green Bio refinery Utzenaich, AT	Organic juice, biogas	Electricity and heat	Aminoacids, lactic acid	Grass, clover grass, lucerne	-	Demo	http://www.energieinstitut-linz.at http://www.fabrikderzukunft.at
Greenmills, NL	Oil, biogas	Biodiesel, electricity and heat	Potassium phosphate, compost	-	Waste oils and fats	Full	http://www.solarix.eu/en/news/112
Inbicon, DK	C6/C5 sugars, lignin	Ethanol, lignin fuel	Animal feed	-	Wheat straw, corn stover	Demo	[28], http://www.inbicon.com
INEOS, UK	Syngas	Ethanol, heat	-	MSW	-	Demo (planned 2012)	http://www.ineosbio.com
Italian BioProducts, IT	C6 sugars, lignin	Ethanol, electricity and heat	-	*Arundo donax*	Wheat straw	Demo (planned 2012)	http://www.chemtex.com/templates/renewables_PROESA.html

Lenzing, AT	C6 sugars	Electricity and heat,	Pulp, cellulose fibres	Wood	-	Full	http://www.lenzing.com
NSE Biofuels Oy,FI	Syngas	Electricity and heat	Cheicals	Wood	-	Demo	http://www.fwc.com/publications/pdf/NSE_Startup_3_25_10_Final.pdf
Pyrobio+, FR	Pyrolysis	Bio-oil, syngas, coke	-	-	Industrial, food, agricultural or MSW	Pilot	http://www.finaxo.fr/Pyrobio-Energy
Pytec, DE	Syngas	pyrolysis oils	-	Wood	-	Pilot	http://www.pytecsite.de
Roquette, FR	C6 sugars, oils	Bioethanol	Cyclo-, malto-, cello-) dextrines, dietary fibers, organic acids, polyols, proteins, starches	Maize, wheat, potatoes, peas	-	Full	[29]
Royal Nedalco, NL	C6 sugars	Ethanol	Feed, vinasse, C5-fermenting yeast	Potato, wheat	Sugar beet molasses	Full (5x)	http://www.nedalco.com/
Sekab, SE	C6 sugars, biogas	Ethanol, electricity, heat	-	Wood	-	Pilot	http://www.sekab.com
St1 Biofuels, FI	C6 sugars	Bioethanol	Feed	-	Potato processing, dairy, bakery and brewery wastes	Demo (3x)	http://www.st1.eu
TMO Renewables, UK	C6 sugars	Bioethanol	-	-	MSW	Demo	http://www.tmo-group.com
VärmlandsMetanol, SE	Gasification	Methanol, heat	-	-	Wood residues	-	
Vitalys, DK	Organic juice	-	Lysine	Grass	-	Full	http://www.agroferm.dk
Vivergo, UK	C6 sugars	Ethanol	Feed	Wheat	-	Full	http://vivergofuels.com
Wabio, DE	C6 sugars, Biogas	Electricity and heat	Fertilizer, CO_2	-	Plant waste (no manure)	Full	http://www.wabio.de

Reference List

[1] World Energy Outlook 2010: Executive summary. 2010;2010. France, International Energy Agency. 23-11-2010.
Ref Type: Report

[2] Kamm B, Kamm M. Principles of biorefineries. Applied Microbiology and Biotechnology 2004; 64(2):137-45.

[3] Cherubini F, Jungmeier G, Wellisch M, Willke T, Skiadas I, Van Ree R et al. Toward a common classification approach for biorefinery systems. Biofuels Bioproducts & Biorefining-Biofpr 2009; 3(5):534-46.

[4] IPCC Fourth Assessment Report, Climate change2007: Working Group III Report "Mitigation of Climate Change": Chapter 5, Transport and infrastructure. Metz B, Davidson OR, Dave R, Meyer L.A., editors. 2007;324-385. Cambridge, UK and New York, USA, Cambridge University Press. 6-9-2010.
Ref Type: Report [5] Directive of The European parliament and of the council on the promotion of the use of the use of energy from renewable sources. Commission, editor. 2008;2008/0016. EU Commission. 9-6-2010.
Ref Type: Report

[6] Carbon Disclosure Project: Transport Report. 2010. London, Carbon Disclosure Project. 22-11-2010.
Ref Type: Report

[7] White biotechnology: replacing black gold? Fifth International Conference on Renewable resources and Biorefineries; 09 Jun 10; Belgium: 2010.

[8] Directive of the European parliament and of the council on the promotion of the use of energy from renewable sources. Commission, editor. 2008;2008/0016. EU Commission. 9-6-2010.
Ref Type: Report

[9] Kamm B, Gruber P, Kamm M. Biorefineries - Industrial Processes and Products. 1st ed. Weinheim: Wiley-VCH; 2006.

[10] Vision for bioenergy and biobased products in the United States. Biomass Research and development Initiative, editor. 2006. 9-6-2010.
Ref Type: Report

[11] Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA et al. The path forward for biofuels and biomaterials. Science 2006; 311(5760):484-9.

[12] von Blottnitz H, Curran MA. A review of assessments conducted on bio-ethanol as a transportation fuel from a net energy, greenhouse gas, and environmental life cycle perspective. Journal of Cleaner Production 2007; 15(7):607-19.

[13] Cherubini F, Stromman AH. Production of Biofuels and Biochemicals from Lignocellulosic Biomass: Estimation of Maximum Theoretical Yields and Efficiencies Using Matrix Algebra. Energy & Fuels 2010; 24:2657-66.

[14] Menrad K, Klein A, Kurka S. Interest of industrial actors in biorefinery concepts in Europe. Biofuels Bioproducts & Biorefining-Biofpr 2009; 3(3):384-94.

[15] Smith W. Mapping the development of UK Biorefinery Complexes. 2007. Tamutech Consuntancy, National Non Food Crops Centre. 7-9-2010.
Ref Type: Report

[16] Rosgaard L, Pedersen S, Meyer AS. Comparison of different pretreatment strategies for enzymatic hydrolysis of wheat and barley straw. Applied Biochemistry and Biotechnology 2007; 143(3):284-96.

[17] Wyman CE, Dale BE, Elander RT, Holtzapple M, Ladisch MR, Lee YY. Coordinated development of leading biomass pretreatment technologies. Bioresource Technology 2005; 96(18):1959-66.

[18] Wellisch M, Jungmeier G, Karbowski A, Patel MK, Rogulska M. Biorefinery systems - potential contributors to sustainable innovation. Biofuels Bioproducts & Biorefining-Biofpr 2010; 4(3):275-86.

[19] Van Ree R. Status report Biorefinery 2007. 2007. 15-11-2010.
Ref Type: Slide

[20] Lyko H, Deerberg G, Weidner E. Coupled production in biorefineries-Combined use of biomass as a source of energy, fuels and materials. Journal of Biotechnology 2009; 142(1):78-86.

[21] European Biodiesel Board. 2010. 20-9-2010.
Ref Type: Internet Communication

[22] Schwietzke S, Ladisch M, Russo L, Kwant K, Mäkinen T, Kavalov B et al. Analysis and identification of gaps in research for the production of second-generation liquid transportation biofuels. 2010;1:1-20. IEA Bioenergy, Task 41.
Ref Type: Report

[23] Neste Oil. Neste Oil to build biodiesel production plant in Porvoo, Finland. Neste Oil Press release 2005 Feb 15.

[24] Naik SN, Goud VV, Rout PK, Dalai AK. Production of first and second generation biofuels: A comprehensive review. Renewable & Sustainable Energy Reviews 2010; 14(2):578-97.

[25] Mandl MG. Status of green biorefining in Europe. Biofuels Bioproducts & Biorefining-Biofpr 2010; 4(3):268-74.

[26] de Mes TZD, Stams AJM, Reith JH, Zeeman G. Methane production by anaerobic digestion of wastewater and soliid wastes. In: Reith JH, Wijffels RH, Barten H, editors. Bio-methane & Bio-hydrogen, Petten: Dutch Biological Hydrogen Foundation, Energy Research Centre of the Netherlands ECN, Unit biomass; 2003, p. 58-102.

[27] Langvad N, Skovgaard-Pletersen R, Mikkelsen MJ. BioGasol's process concept for production of cellulosic ethanol - status and perspectives. International Sugar Journal 2010; 112(1334):104-9.

[28] Larsen J, Petersen MO, Thirup L, Li HW, Iversen FK. The IBUS process - Lignocellulosic bioethanol close to a commercial reality. Chemical Engineering & Technology 2008; 31(5):765-72.

[29] Clark JH, Deswarte FI. Chemicals from biomass. 1st ed. Wiley and sons; 2008.

2 AN OILSEED RAPE WHOLE-CROP BIOREFINERY: A CASE STUDY

2.1 Introduction

In the following paragraphs, the idea of expanding an existing rapeseed biodiesel plant into a complex whole-crop oilseed rape biorefinery is presented and discussed. This scenario exploits the major macro-components of oilseed rape for production of chosen fuels, energy, chemical building blocks, and enzymes.

Oilseed rape is comprised of the macro-components carbohydrates, fats, proteins, lignin, and micro-components like waxes, tannins, ashes, and glucosinolates. Thus, a variety of foods, feeds, fuels, chemicals, fibres, and energy can be extracted. The targeted products of the biorefinery have been chosen based on 1) priority to Low value-High Volume (LVHV) products like commodity chemicals 2) ability to cover an established market niche (preferably in the energy sector); 3) the product yields; 4) the technological robustness of the relevant production methods [11] allowing speedy production start, i.e. by 2020 in Europe; 5) the material and energy integration, and process flexibility; 6) a preference has been given to low temperature biological processes with high productivity. Even under these restrictions, numerous products and processes can be combined.

In Cherubini terms [16], a *"One platform (oil) biorefinery for biodiesel, glycerol, and animal feed, from rapeseed"* will be upgraded to a *"Four platforms (C6 sugars, biogas, lignin, and oil) biorefinery for biodiesel, chemical building blocks, ethanol, electricity and heat from oilseed rape".*

2.2 Oilseed rape plant

Oilseed rape plant is a well known oil-rich crop, cultivated for its extractable oil, which is used mainly as food and for production of biodiesel or oleo-chemicals [18]. In 2009, 5 million hectares (Miha) were cultivated in EU and 21.4 MiT of rapeseed and 31.2 MiT of rape straw was harvested. From rape

seed 45% wt. oil and 55% wt. de-oiled cake is obtained. Presently, only the oil fraction (18%wt. of the whole plant), is used by the food and biodiesel industries, while the residues rapeseed cake, rape straw, and glycerol (from biodiesel production), have only low-grade applications in the market, like for example animal fodder, or burning fuel.

Oilseed rape (*Brassica Napus L.*) is a member of *Brassiceae* family, part of bivalves group (*Dicotylidoneae*). Rape has three distinctive species, *Brassica nigra, Brassica oleracea*, and *Brassica campestris*. In Europe, the most widely used variety [20] is winter rape of double low variety (double zero, 00, or canola, containing low erucic acid and low glucosinolates content). Other known varieties is the wild type (HEAR), or triple low (yellow seed variety containing low erucic acid, low glucosinolates, and low fiber) that is gaining popularity the last years. Oilseed rape grows even in sandy soils of varying pH, but maximum yield is obtained in heavy clay soils [21] at high level of nitrogen fertilizer, accounting for 70% of energy input in the field [22].

2.3 Rapeseed line

2.3.1 Pretreatment

In industry, rape seed is pretreated by flaking and cooking before oil is extracted either by mechanical pressure and/or chemical assisted extraction with e.g. hexane [12,23]. The process of pretreatment is illustrated in Figure 2. The filtrate contains in addition to oil (tri-acyl-glycerides, TAG), also small amounts of free fatty acids (FFA), water, gums, lipids, sterols, and other impurities [23]. Extracted oil needs to be degummed (phospholipids) [24], and refined, depending on the further processing route, to biodiesel [12]. The de-oiled cake is separated from the solvent, is mixed with gums and the mixture is toasted. Presently, the biodiesel industry in Denmark consumes 90% of the produced rapeseed oil. The technology of pretreating rapeseed and conditioning of oil is mature and in many cases separate

from biodiesel plants. A process scheme for pretreating rape seed is presented in Figure 2.

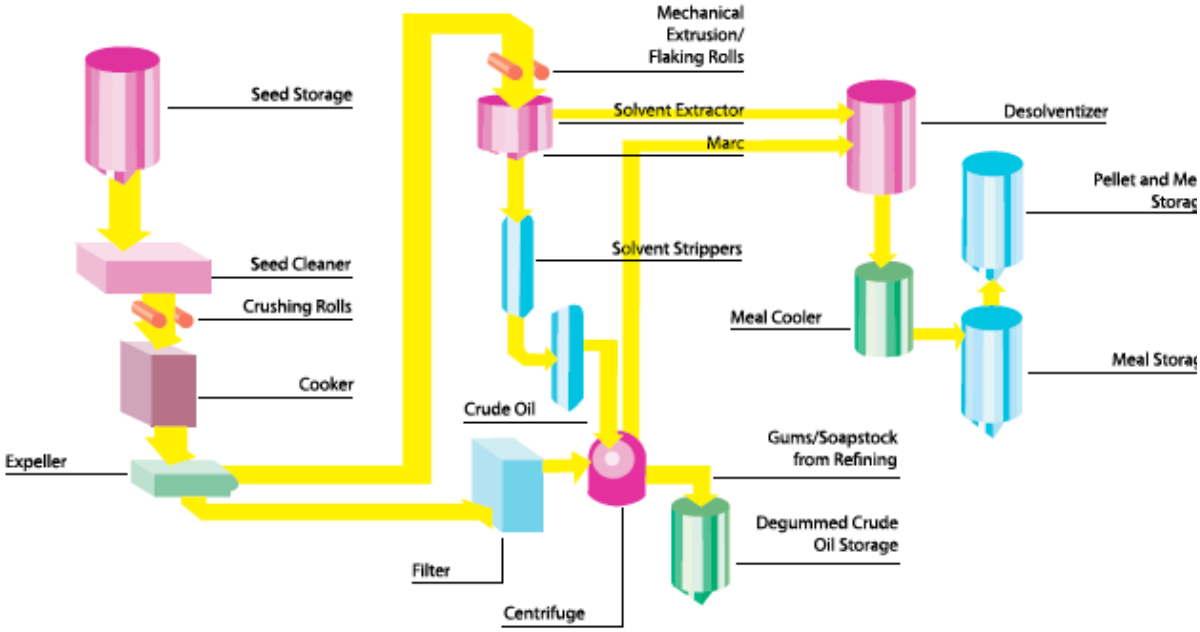

Figure 2 Schematic representation of rapeseed pretreatment [1]

2.3.2 Biodiesel from rapeseed oil

Biodiesel is a mixture of alkyl-esters (usually methyl- or ethyl-esters,) of fatty acids and has quite good transport fuel properties [25]. Production of biodiesel has been carried out in full scale for the past 20 years and the industry is mature. In Europe there are about 245 existing biodiesel plants [26], most of which use rapeseed oil as feedstock and have a production capacity of 10-50 million gallon/yr. [27]. Presently biodiesel is available at petrol stations in most EU countries.

Biodiesel is produced by transesterification of tri-acyl-glycerides (TAG) of oil with methanol or ethanol, with the help of a catalyst. Four transesterification methods to produce biodiesel exist: 1) with alcohols and alkaline (homogeneous or heterogeneous) catalyst, 2) with alcohols and acid (homogeneous or heterogeneous) catalyst, 3) non-catalyzed with supercritical methanol [17], and 4) bio-catalyzed using lipases in

combination to alcohols [28] or supercritical CO_2 [29]. Typical technologies for production of rapeseed biodiesel coupled to the process conditions and produc yields are presented in Table 4.

Table 4 Most common rapeseed biodiesel production technologies

Type of catalysis	Catalyst	Catalyst amount (%)	Alcohol, alcohol to oil ratio	Reaction conditions	Ester yield (%)	Ester conversion (%)	Ref.
Alkaline soluble catalyst	KOH	1	MeOH, 6:1	65 °C, 2 h	96	-	[9]
Solid catalyst	KF/Eu_2O_3,	3	MeOH, 12:1	65 °C, 1 h	-	92.5	[14]
Non catalytical	-	-	MeOH, 42:1	350 °C,45 MPa, 4 min	-	95	[17]
Lipase	Source: *Candida Antarctica*, homogeneous	3	MeOH, 4:1	35 °C, 12 h, t-BtOH solvent	95	95	[19]

The most common industrial method for production of biodiesel from rapeseed oil is alkaline assisted catalysis using NaOH or KOH and with methanol as reagent. This leads to fatty methyl-esters and the production in Europe is 500-10,000 T/y [30] and is usually produced in batch mode. It is estimated 1,000 kg of degummed and de-acidified rapeseed oil produces 1,000 kg biodiesel, 180-190 kg crude glycerol [31] and 200 g wastewater [32]. Alcohol is added in surplus (minimum 4:1) and is recycled by distillation. A simplified scheme of the industrial process is presented in Figure 3 [12].

In the future, it is expected that biodiesel production technology will be updated to a continuous system to increase production capacity. This requires major changes in reactors, separation systems, pipings etc, as well as an immobilized/insoluble catalyst to replace soluble catalysts whether inorganic or organic. This will lower the cost of catalyst and also reduce the costs of oil purification [30]. Currently, only a few plants operate with solid catalysts. Diester Industrie in France uses the “Esterfip-H” solid catalyst

technology, which operates without downstream washing steps and provides an oil yield exceding 99% and salt-free glycerol of 98% purity [17,33]. Tan et al. [34] has presented two scaled up projects in China that utilize enzyme catalyst: 1) Lvming company, based in Shanghai, uses waste oil at 10,000 T/y capacity with an immobilized lipase from *Candida sp.* as biocatalyst and obtains a 90% yield; and 2) Hainabaichuan company in Hunan province with lipase Novozyme 435 from Novozymes.

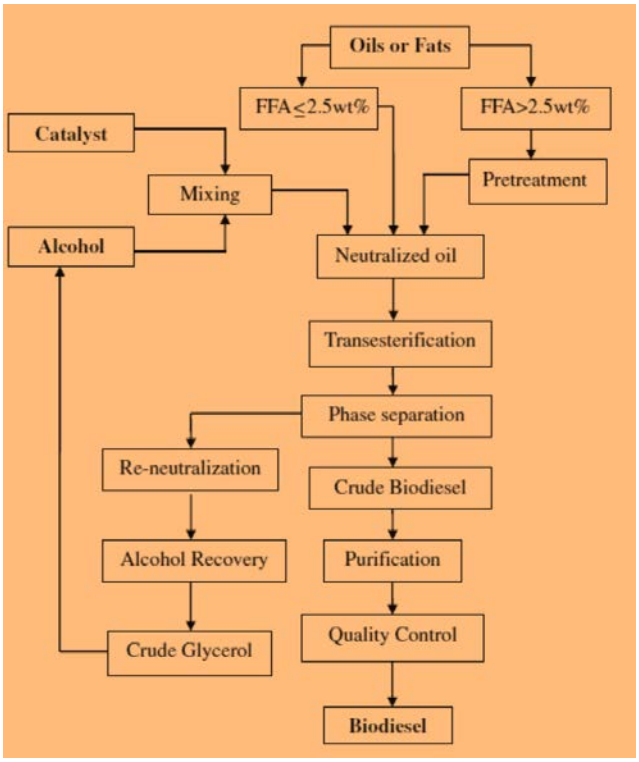

***Figure 3 A simplified scheme of alkaline catalyzed biodiesel production* [12]**

The incorporation of heterogeneous catalysis for biodiesel needs refinement to gain popularity over conventional KOH catalysts. One of the major drawbacks of insoluble catalysts as compared with soluble catalysts is low productivity, requiring higher temperatures [30] (see Table 4). At present, alkaline catalysis gives the highest yields and the highest productivities, but the method is characterized by serious limitations with low quality of oil. Alkaline catalysis requires oils that have negligible content in FFA (<0.3%

[24]) and water (<0.05%[35]) because both consume the alkaline catalyst. Virgin rapeseed oil contains about 1.5 % %wt. FFA [36], necessitating removal or esterification of the FFA's [12], or alternatively, use of another catalyst such as a lipases[37].

Lipase is the best catalyst in processing low quality oils with high water and high FFA content. Lipases esterify FFA into biodiesel, are water-friendly [37]. Also, purity of produced is high [37] and washing is of produced is not necessary. With enzymes reaction rates can be relatively fast (see in Table 4 and Table 5, and [37]), but high costs of production and immobilization is a limitation. According to Sotoft et al. [38] production of rapeseed biodiesel by lipases is economically viable for high production scales (200,000 T/y) with present technologies and prices of raw materials.

The choice of acyl-acceptor has a major technical and economical impact on biodiesel production. Methanol is preferred by industry due its low price, and higher reactivity with inorganic catalysts [39]. However, methanol is traditionally produced by fossil sources, which undermines renewability of fuel [40]. Only recently, "green" methanol entered the market; BioMCN in the Netherlands developed a commercialized a technique that produces methanol from glycerol using Fischer-Tropsch (FT) technology (see Table 3). However, if biobased methanol is not an option bioethanol can function as acyl-acceptor. Produced ethyl-esters have heating values (40.7 GJ/T) that exceed methyl-esters (37.8 GJ/T), and production yields are good (See Table 5).

Table 5. Ethanol biodiesel literature findings

Substrate	Catalyst	Catalyst amount (%)	alcohol to oil ratio	Reaction conditions	Ester yield (%)	Ester conversion (%)	Ref.
Palm oil	Lipases from *Pseudomonas fluorescens,* immobilized on POS-PVA and activated by glutaraldehyde	20% of volume of reactans	18:1	58 °C, 24 hours	-	91	[10]
Cynara cardunculus L.	NaOH	1%	12:1	75 °C,	-	94.5	[15]

2.3.3 Chemical building blocks from glycerol

Glycerol is an impure byproduct of the biodiesel industry, produced in 10 % wt. ratio to biodiesel. Lack of processing industries and/or end-market uses has created an oversupply of glycerol in the market for the last five years [12]. In EU 900,000T/y of glycerol is produced [26]). Purified glycerol is consumed in cosmetic, beverage, personal care, pharmaceutical and material end markets, but the supply is much bigger, and as a result, crude glycerol of any grade in 2007 has price of only $0.005/lb or lower [41].

Glycerol is a three-carbon (C3) building block part in NREL's list of 12 Top value added chemicals from biomass [42]. Figure 4 shows some of the possible intermediate products from glycerol [7]. Many reviews have recently presented how glycerol can be converted to useful products by chemical processing [7,43] or fermentation [44].

Industrial scale processes that use the abundant glycerol are rare. Novel promising technological routes for exploitation of glycerol are the following.

A new production method that converts glycerol to propylene glycol via catalytic hydrogenation [43,45] has resulted in a 10-fold reduction in the production costs as compared with petrochemical route[46]. Propylene glycol (PG) has a promising market of 450,000T/y and Archer Daniel Midland (ADM) has announced (2009)the building of the first bio-PG plant in USA with a capacity of 100,000 T/y [47].

Epichlorohydrin is a well-marketed petro-chemical product that is used in epoxy-resins, and has been reported to activate immobilized lipases used for biodiesel production [10]. Solvay in France is currently building a full scale plant of 100,000 T/y in Thailand [48] for production of epichlorohydrin from glycerol via "Epicerol" technology.

Finally, as mentioned above, production of methanol from crude glycerol using cracking and Fischer-Tropcsh (FT) technology has been scaled up by BIO-MCN to a 200,000 T/y full scale plant in the Netherlands [49]. Given the encouraging economy of FT technology for methanol production [11]) and

the tremendous potential of biodiesel industry in the future, it is expected that this technology will become popular.

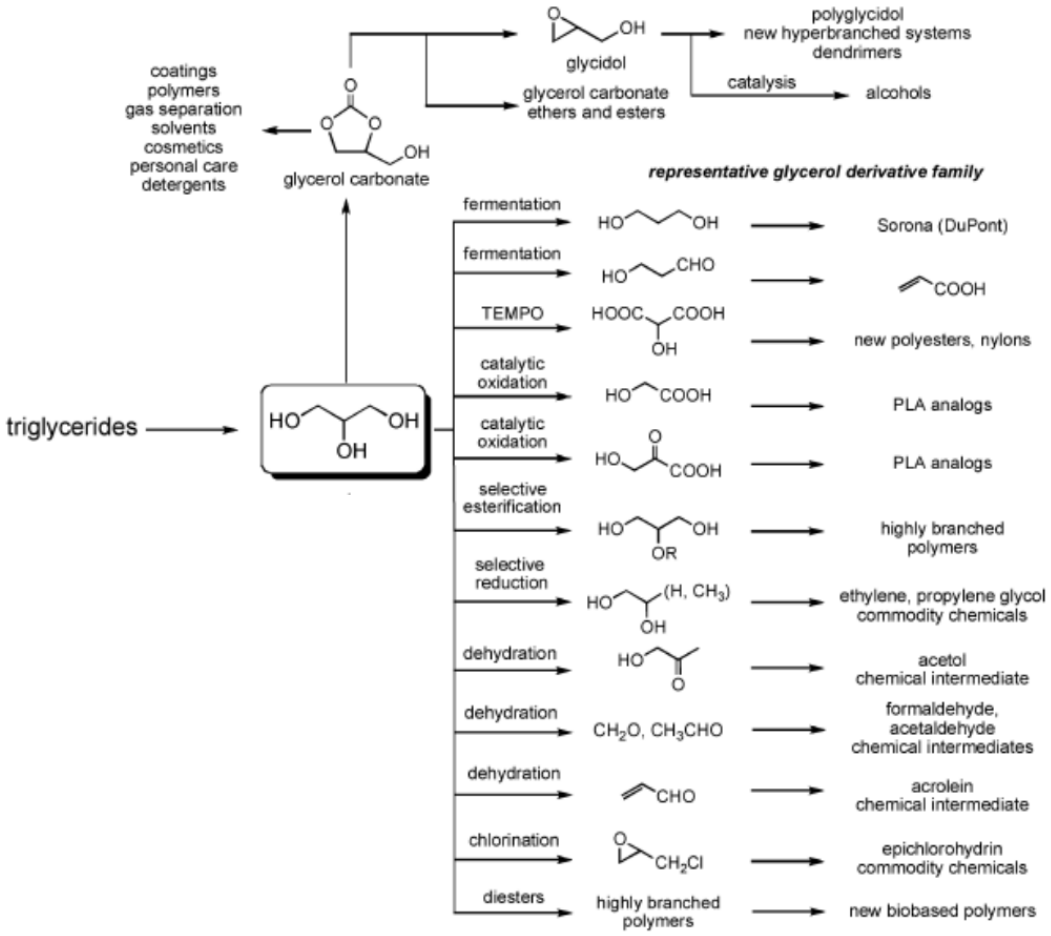

Figure 4 Biorefining the glycerol potentials into added-value products, adapted by Bozell et al. (2009) [7]

2.3.4 Animal fodder from rapeseed press cake

Rapeseed press cake (or rapeseed extraction meal) is the solid residue after pressing out the oil. It s estimated that 10.8 MiT of rapeseed cake (calculated from FAOSTAT data) was produced in 2009, mainly from Canola. Canola cake has currently low-grade uses like burning for production of energy (20.8 MJ/kg [50]), organic fertilizer, or animal feed (second in global consumption after soybean cake [51]. It has excellent nutritional value, and is used as meal for mono-gastric (hen, turkey, pig,

horse, rabbit) and ruminant animals (sheep, cattle) [3], and in aquaculture [51].

Canola meal contains 38% protein and 18% carbohydrate [3]. The carbohydrate fraction consists of pectin (14.5%), cellulose (7%), lipids (5%), soluble sugars (5%), starch (4.5%), arabinan (2%), and arabinogalactan (1%) [52] The canola meal contains a high proportion of essential sulfuric and aromatic amino acids as well as minerals like Ca, Mg, and P, vitamins B4 and E [20]. However, canola meal contains some anti-nutritional components that lower the quality of the meal for some animals, e.g. pigs [51]. Development of good quality rapeseed meal for livestock in EU will reduce the import of soybean meal from Argentina and Brazil [53], and decrease the GHG emissions from overseas transportation [54]. Canola meal contains low amounts of erucic acid (<2%) and glucosinolates (less than 8 μmol/g cake), as well as phytic acid (limits phosphorus bioavailability [55]), sinapate esters (phenolic acids, bad taste [20]), tannins (condensed phenolics) and crude fiber, both giving dark seed, that decrease the digestibility and increase toxicity of the meal [3]. Widespread utilization of fodder to both monogastric and ruminant animals requires reduction of the toxicity and increase of digestibility of proteins and nutrients. Use of enzymes like phytase for improvement of the digestibility is common practice, and it is considered to add cellulases and hemicellulases for the same reason. Alternatively, improvement of quality of rapeseed meal could be achieved by exploitation of plant breeding methods that will produce varieties with lower amounts of anti-nutrition elements [3].

The canola variety that is almost exclusively cultivated in Northern Europe [20] has been produced by breeding and natural mutation [20]. A new yellow seed variety, called triple zero variety, is becoming more and more popular because of the high digestibility of the cake: 30% higher for energy, and 20% higher for protein [3] as compared with the canola meal. Comparison of composition of yellow and brown rapeseed is illustrated in Table 6. The increase in digestibility with the yellow variety is probably due to higher content of protein and lower content of tannins, crude fiber, and lignin [3]. The oil content is the same for both varieties [20].

Considering the abundance of rapeseed cake and its value as nutrient alternative applications should be considered, e.g. as a medium for many biotechnological applications [56]. Koutinas et al. (2004) examined the possibility of using rapeseed cake as a medium for cultivating *Saccharomyces ceresiviae* [57] and Ramachandran et al. *[56] in the review* reported production of cellulases and hemicellulases by *Trichoderma resei*.

Table 6. Composition of brown and yellow rapeseed fractions (modified by Bell *[3]*)

	Brown seeds (% oil-free DM)			Yellow seeds (% oil-free DM)		
	Seed	Hull	Embryo	Seed	Hull	Embryo
Crude protein	38.29	13.2	56.7	NA	17.8	51.0
Crude fiber	11.5	44.4	3.5	7.1	25.6	3.1
Lignin	10.5	18.5	4.9	5.4	14.8	6.0
Polysaccharides	17.7	NA	NA	21.2	NA	NA
Ash	NA	7.0	4.3		5.9	7.1

2.4 Rape Straw Line

In the following paragraphs technologies and products for rape straw biorefinery will be presented and discussed based on literature review and my own experimental findings in pretreatment, ethanol production and enzyme production.

2.4.1 Pretreatment

The earliest biomass pretreatment was produced for the pulp and paper industry, *"One platform biorefinery (C6 sugars) for production of paper from wood"* that was analyzed in Chapter 1.4. Traditionally pulp and paper industry uses chemical or thermo-chemical methods to remove lignin (a phenyl-propane 3D polymer) and hemicellulose (heteropolymers of C5 and C6 sugars, branched extensively by acetic acid) from cellulosic fibers

(highly packed homopolymer of glucose) for paper production. Nowadays, pretreatment is a prerequisite for (bio)processing lignocellulose into different products, and this step is used in most industrial ethanol plants processing lignocellulose like wood, bagasse, straw (see Figure 5 for global view).

The purpose of pretreatment is to increase the digestibility of lignocellulose by increasing the bioavailability of the carbohydrates (cellulose and hemicellulose) for microbial conversion [58]. This is accomplished by loosen up the structure of cellulose [8,59] and removing part of the lignin and hemicellulose coatings [58,60]. During this process these components are degraded to some extent as is also seen with the pulp and paper pretreatment techniques. In complex biorefineries, pretreatment is not designed to degrade lignocellulose to a high extent. In the biorefinery presented here, rape straw is the feedstock for a biorefinery consisting of three platforms, and all three major components (cellulose, hemicellulose and lignin) have value as precursors for products. In other words, in the context of this project, pretreatment should enhance the digestibility of rape straw, but should also preserve and recover both hemicellulose and lignin in a useful form.

The Iogen demo plant in Canada is a *"two platforms C6 sugars and lignin biorefinery for bioethanol, and heat production from lignocellulosic biomass".* Pretreatment is carried out by chopping and milling wheat straw followed by 1) a thermo-physico-chemical method using high pressure steam and dilute sulfuric acid (0.5-2%) at high temperature (180-260 ^{o}C) for a short time (0.5-5 min) and 2)abrupt release of pressure [61]. The technique is called "Steam Explosion".

Inbicon in Denmark (see Table 3) uses a *"three platforms C6/C5 sugars and lignin biorefinery for bioethanol, fodders, and heat production from lignocellulosic biomass",* that utilizes IBUS technology [62]. In this process biomass (like wheat straw) is chopped and then pretreated by steam at high pressure and high temperature (195 ^{o}C) for 15 minutes. The method is called "Hydrothermal Treatment".

Finally, BornBiofuel 2 biorefinery is under construction in Denmark. It is *"a three platforms C6/C5 sugars and lignin biorefinery for ethanol, biogas and*

heat from lignocellulosic biomass" that twill upscale Maxifuel technology. In the Maxifuel process, biomass is subjected to wet oxidation (a submerged method using oxygen) in combination with "Steam Explosion", in total termed "Wet Explosion" [63].

A number of pretreatment methods for lignocellulosic biomass are being considered [58,64]. The assessment of the efficiency of the pretreatment is based on numerous indicators like sugar yields that are produced by enzymatic hydrolysis of carbohydrates, and ethanol yields after enzyme saccharification and fermentation. Each pretreatment method has its pros and cons [63], and its effectiveness depends on the properties and structure of feedstock. The mean composition of three varieties of rape straw are listed in Table 7, and the structure of cell wall is shown in SEM pictures in Figure 6.

Table 7 Average composition of three varieties of rape straw (Bienvenu, Rafal, and Jen Neuf) (adapted by Alexander wt al. [8]) After ±the standard deviation is given.

Component filter-cake	Concentration (g/100g WIS)
Rhamnose	0,5 ±0,1
Fucose	0,1 ±0
Arabinose	0,9 ±0,4
Xylose	16,5 ±0,6
Mannose	2,9 ±0,3
Galactose	1,9 ±0,3
Glucose	41,3 ±2,8
Uronic acids	7,5 ±1,1
Total phenolics	9,5 ±1,0
Nx6.25	6,2 ±2,8
Acetyl groups	3,0 ±0,2
Ash	5,4 ±0,7
Ether extractive	3,0 ±2,2

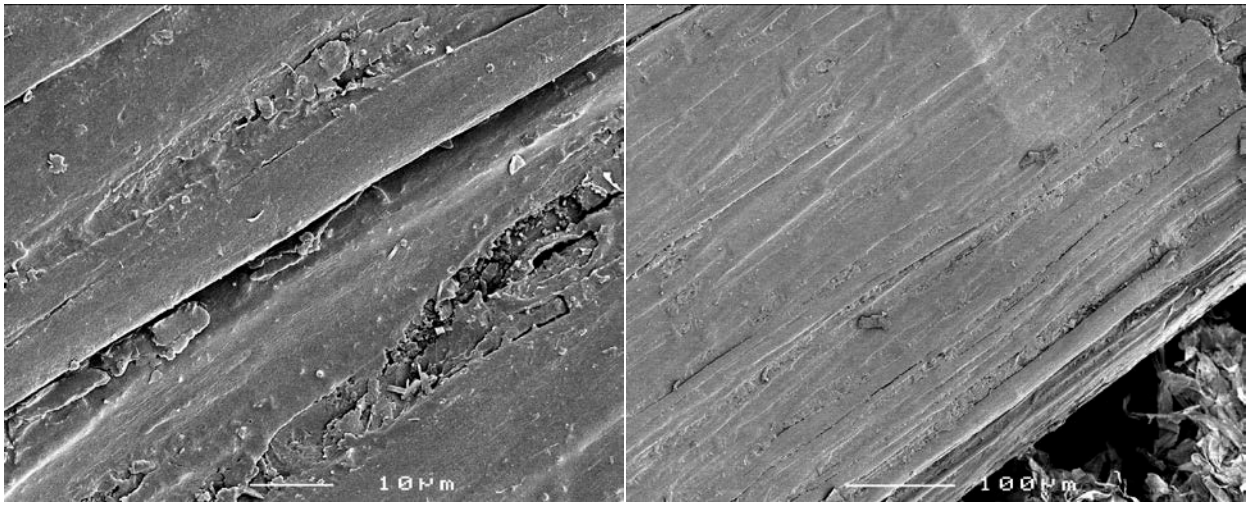

Figure 6 Scanning-electron microscopy pictures of rape straw fibers [4]

Rape straw has not been studied extensively for production of added-value products. In Table 8, all literature studies on pretreatment technologies of rape straw are presented. Lu et al (2009) pretreated rape straw with dilute sulfuric acid [65], Li et al. (2009) tested the effect of a two-step chemical-rich pretreatment method using acetone with phosphoric acid that saccharified more than 99% of the cellulose [66]; Two investigations using hydrothermal treatment have been reported and both caused severe degradation of the cellulose [67,68]. Finally, a biological treatment using the delignifying white-rot fungus *Pleurotus ostreatus,* for production of mushrooms has been investigated *[69]*.

The fate of hemicellulose and lignin will determine the properties of pretreatment [58,64]. Qualifications of a pretreatment method sought for ethanol production are: maximum recovery of sugars, complete and fast digestibility of pretreated rape straw by enzymes and yeasts (high product yields and productivities), and low economy (energy, water, chemicals) [58,70]. Economy is very important parameterer because pretreatment is often capital intensive and energy consuming [71]. In a biorefinery with low-value-high-volume (LVHV) products, like the one presented here, the window of profit is very small, and therefore the pretreatment should be economic and efficient [72-74].

Wet oxidation (WO) is a low cost pretreatment method and initial studies have previously been carried out with rape straw [75]. WO pretreatment has been tested with several other starting materials (see Table 8) where high recoveries and product yields have been recorded [76].

In **Paper I** pretreatment of rape straw by WO is optimized for rape straw for bioethanol production. The effect of reaction temperature, reaction time, and oxygen gas pressure was investigated for maximum ethanol yield. Moreover features of pretreatment were tested for reducing the water use and increasing the energy efficiency like recycling liquid (filtrate) in WO, presoaking biomass in water or recycled filtrate before WO, skipping washing pretreated solids (filter cake) after WO, or using whole slurry for ethanol production. Results showed that short WO treatments (2-3 minutes) produced higher ethanol yields and sugar and lignin recoveries, than longer WO (15 minutes). The highest obtained ethanol yield was 67% after fermenting the whole slurry produced by WO at 205 ^{o}C for 3 min with 12 bar of oxygen gas pressure featured with presoaking in water. At these conditions after pre-treatment, cellulose and hemicellulose was recovered quantitatively together with 86% of the lignin.

Table 8. Summary of findings from literature review and data from lab experiments (last line) of processing several feedstocks by WO, and subsequently enzyme saccharification and/or SSF for ethanol production. The numbers in parenthesis refer to the convertibilities of processing the whole slurry

Feedstock	Pre-treatment conditions	Enzyme hydrolysis conditions	SSF conditions	Glucose yield (%)	Ethanol convertibility (%)	Reference
Wheat straw	WO 195 °C,15 min, 12 bar O_2, 6.5 g/l Na_2CO_3, 6% DM;	2% DM, 30 FPU/g DM Celluclast, Novozym 188, 50 °C/24 hours;	10 % DM, 25 FPU/g DM Celluclast, Novozym, 50°C/24 hours and 32°C/148 hours, *baker's yeast* 3.2 g/l	70 (66)	-(70)	[77]
Corn stover	WO 195 °C,15 min, 12 bar O_2, 2 g/l Na_2CO_3, 6% DM	% DM, 25 FPU/g DM Celluclast, Novozym 188, 50 °C/24 hours	-	83 (64)	-(-)	[78]
Sugarcane bagasse	WO 195 °C,15 min, 12 bar O_2, 2 g/l Na_2CO_3, 6% DM;	% DM, 25 FPU/g DM Celluclast, 0.46 CBU/ml Novozym 188, 50 °C/24 hours	-	64 (56)	-(-)	[79]
Spruce[e]	WO 200 °C, 20 min, 12 bar O_2, H_2SO_4 for pH 3.5, 6% DM;	2% DM, 30 FPU/g DM, Celluclast, Novozym 188, 50 °C/24 hours	-	53(-)	-(-)	[80]
Clover/Rye grass mixtures	WO 195 °C, 10 min, 12 bar O_2, 6% DM	2% DM, 25 FPU/g DM Celluclast, 0.46 CBU/ml Novozym 188, 50 °C/24 hours	10% DM, 35 FPU/g DM Celluclast, Novozym, 50°C/24 hours and 32°C/163 hours, baker's yeast 2 g/l	76 (75)	-(87)	[81]
Household waste/wheat straw[g]	WO 195 °C, 10 min, 12 bar O_2, 6% DM	2% DM, 25 FPU/g DM Celluclast, 0.46 CBU/ml Novozym 188, 50 °C/24 hours	10% DM, 35 FPU/g DM Celluclast, Novozym, 50°C/24 hours and 32°C/192 hours, baker's yeast 1.6 g/l	72 (78)	70(-)	[82]
Rape straw	Wet Oxidation, 6% DM, 12 bar O_2, 2g/l Na_2CO_3, 195 °C, 15 min	2% DM, 25 FPU/g DM Cellubrix, 50 °C/24 hours	Whole slurry 12.5% DM, 35 FPU/g DM Cellubrix L, 3 g/l baker's yeast, 0.8 g/l urea	58	61[1]	[75]
	Soaking 80 °C/20 min, WO 205 °C, 3 min, 12 bar O_2, 6% DM	2% DM, 25 FPU/g DM Cellubrix, 50 °C/24 hours;	Whole slurry 12.5% DM, 35 FPU/g DM Cellubrix, 50°C/24 hours and 37°C/116 hours, *dry baker's yeast* 2.5 g/l	55(48)	66(65)	**Paper I**

2.4.2 Ethanol from rape straw cellulose

Current status of use of the 31.2 MiT of rape straw produced in EU in 2009 is low grade applications. Rape straw is currently burned at Combined Heat and Power (CHP) plants in Denmark [83] (16MJ/kg [50], but compared to other straws rape straw contains high content of ash [84]. While other uses of rape straw are fodder [8], or soil conditioner [54,85,86].

Given the broad utility of C6 sugars by microorganisms, C6 fermentations for food, feed, materials, chemicals, and fuels have many possibilities [87]. Returning to the criteria set for the product selection for the biorefinery of Chapter 2.1, priority is given to biofuels with proven technology and with available markets. Ethanol and biogas are the most popular biofuels worldwide with ethanol about 68 BiT production [88], and biogas with 5,9 MiT for 2010 in EU [89]. For these reasons, ethanol and biogas were the strongest candidates. Finally ethanol was prioritized to biogas, due to higher productivities [90].

Production of ethanol from cellulose has been studied extensively in literature and in the last years demonstration plants have been built all over the world, (see Figure 5). Cellulosic ethanol full scale plants are expected in a few years (2013: the Abengoa Bioenergy Plant; POET Energy's Project Liberty Plant; and Great River Energy's plant). In Denmark, Spain, Canada, and the USA, ethanol has already been marketed reaching car tanks, branded as E5, E10, or E85. However, rape straw has not been studied adequately for ethanol production.

Conventional technology for cellulosic ethanol is as follows: After pretreatment that was described above, cellulose is hydrolyzed by hydrolytic enzyme systems (cellulases, hemicellulase) into glucose [91], which is then fermented into ethanol by yeast. Finally ethanol is distilled by fractional distillation, dehydrated and sold in the market. Nowadays, *Saccharomyces cerevisiae* is the working horse of industrial ethanol production, but its main drawback is that wild strains cannot ferment C5 sugars [92]. In **Paper III**, wet-oxidised (WO) rape straw whole slurry was optimised during Simultaneous Saccharification and Fermentation (SSF) experiments by testing three pure

cultures of *S. cerevisiae* and a baker's yeast. Parameters in focus were thermotollerance up to 40 °C, low pH, and increased DM up to 18%. Results showed no significant difference in the ethanol yields with three pure cultures at 32 °C (70-75%) and 37 °C (72-76%). However, SuMo (isolated from baker yeast) and Turbo (isolated from a brewing yeast formulation) achieved higher ethanol yields at 40 °C (64-66%), after 115 hours of SSF. Increasing DM from 12.5% to 16% DM during SSF experiments with SuMo yeast at 37 °C for 120 hours did not have any significantly influence on the ethanol yields (75-76%).

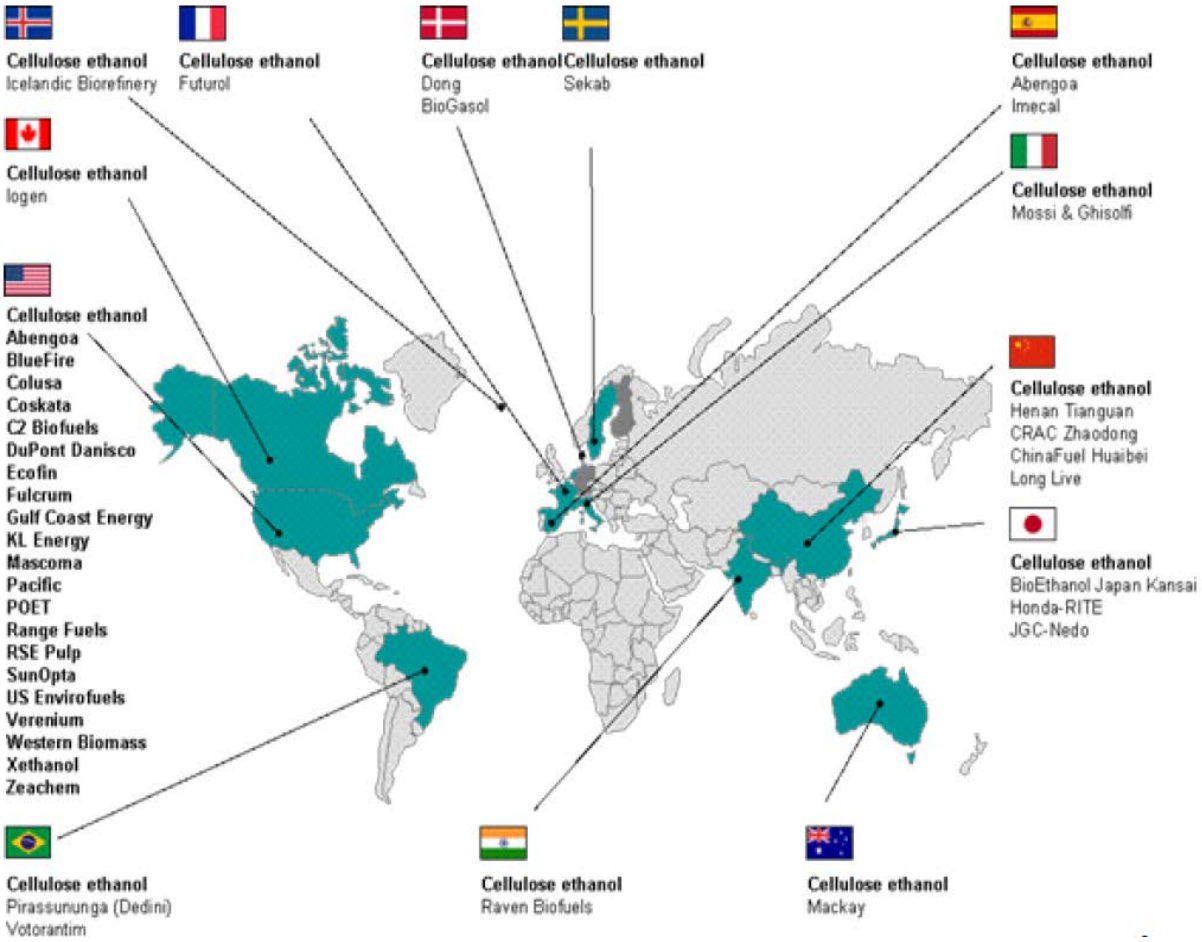

Figure 5 World cellulosic ethanol projects and plans, demo and commercial scale for 2010 (Source: Ecofys [2])

Current conventional ethanol production system from lignocellulose biomass like rape straw has many economic weaknesses, and is expected to be upgraded in the future. By use of biotechnological tools [93,94], it is expected that strains able to secret enzymes and ferment efficiently all sugars, will prevail in industrial scale [95]. So far, there is a considerable activity in R&D and pilot scale for producing efficient ethanol yeasts and bacteria that are co-fermenting C6/C5 sugars for SSF. Nowadays, such technology is taking off branded by companies like Taurus Energy (Sweden), Nedalco (Netherlands), BioGasol (Denmark), and TMO Renewables (UK).

2.4.3 Cellulolytic enzymes from pretreated rape straw

Cellulases are a group of enzymes with various applications in industry like textile, food, wine, brewery, chemicals, feed, pulp and paper, agriculture, and fuels [96,97], and they are playing a key role in lignocellulosic biorefineries [98]. In hereby biorefinery, cellulases facilitate hydrolysis of cellulose and (partially) hemicellulose into monomers (C6 and C5 sugars). Enzyme producing giants in the market are Novozymes, and Danisco [99], which in early 2010 launched new special products for saccharifying lignocellulosic biomass (a total of two cellulase formulations, two hemicellulase formulations, and one mixture). According to their campaign these enzymes are working more efficiently and are avaukable at low costs [100,101]. Before, the available cellulolytic and hemicellulolytic enzyme products were too expensive and too unspecific to cope with the complexity and recalcitrance of lignocellulosic substrates, as well as follow the economic demands of cellulosic ethanol production.

Alternatively to purchasing cellulases, it is possible to produce them onsite by various carbon sources through enzyme fermentation [102]. Such a concept is applied by logen biorefinery in Canada and in Chemrec in Germany. In logen, necessary cellulolytic enzymes, for hydrolysing biomass for ethanol production, are produced onsite by *Trichoderma spp.* and pure sugars, and used readily in the biorefinery [61]. While in Chemrec a part of the

lignocellulosic biomass feedstock of the biorefinery is used for production of cellulases that are then feeding the ethanol production line.

On-site enzyme production in the oilseed rape biorefinery 1) allows control of the quality of enzymes used; 2) provides independence from unsecured enzyme market price and 3) can achieve competitive production costs. Currently there are only 5 enzyme products from big enzyme producers for a vast amount of biomasses. It is questionable if these formulations are the optimized for rape straw cell wall composition and structure (cellulose, hemicellulose, and lignin). In view of the necessity of tailor-made enzyme products, already some enzyme producing companies like AB Enzymes or Proteus provide services for onsite production of tailor-made enzymes to biorefineries. The enzyme production costs depend mainly on substrates cost, the design and the size of enzyme production plant, and the wastewater treatment [103-105]. In the case low cost carbon sources are used like rape straw or rapeseed cake, production costs can be considerably low. Finally, a considerable cost reduction is expected with onsite enzyme production, by skipping the unnecessary downstream purification and formulation.

The quality of carbon source in enzyme fermentations very much holistically determines the final quality and the proportion of distinctive enzyme identities in the final enzyme cocktail product [106,107]; and improved sugar yields have been observed when the same biomass is used as carbon source for the enzyme production and as enzyme substrate [108]. Because of this possible correlation, many complex carbon sources have been tested for production of cellulases [106,109-111]. To the best of the knowledge of the author, rape straw has never been examined for production of cellulases.

In **Paper II** the best enzyme mixtures for hydrolyzing cellulose and hemicellulose in pretreated by Wet oxidation (WO) rape straw are investigated. For this, available commercial enzyme mixtures from Novozymes, as well as bench-scale produced enzymes from *T. reesei* with pretreated rape straw as carbon source are tested. Four fractions of WO pre-treated rape straw: (1) moist filter cake, (2) whole slurry, (3) partly detoxified whole slurry, and (4) partly detoxified filtrate were used as carbon source for

Trichoderma reesei for enzyme production. All enzymes were then tested with a standard activity test with WO pretreated rape straw as substrate, and sugar yields were measured.

The significantly highest glucose yield was achieved for the commercial enzymes Celluclast and Cellubrix (77-79% of the theoretical possible). Among the produced in the lab enzymes, the highest glucose yield was achieved with enzymes produced with the whole slurry of WO pretreated rape straw as carbon source ranging 71-72% of the theoretical. Among the enzyme production experiments, the whole slurry of WO pretreated rape straw induced the highest filter paper activity yields 123 FPU/g monosaccharide equivalents (at 11 days of fermentation). For this enzyme production setup it was estimated that if 100% of biomass used in the biorefinery, about 20% of biomass needs to be allocated for enzyme production (carbon source), while the remaining 80% can be used for bioethanol production.

Before enzyme production from rapes straw is integrated in the oilseed rape biorefinery by 2020, the process should be optimized and scaled up in collaboration with an enzyme company specialised in onsite tailor-made enzyme production.

2.4.4 Chemicals and energy from lignin

Lignin acts as the glue of the plant cell wall and it comprises of a 3D network of C9 phenyl-propane units, mainly of p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol condensed units, the ratio of which greatly depends on plant species. Information on the composition of rape straw lignin is not available, but lignin from grasses is generally dominated by coniferyl- and sinapyl- units. Lignin is a an exploited by-product in pulp and paper industry that is typically burned onsite (LHV 27 GJ/T [112]) for production of energy for the pulp and paper plant. Lignosulfonates are sulfonated lignin derivatives that *are commercialized by a few pulp and paper* plants worldwide with $600M turnover in 1996, as building materials, plasticizers, resins, adhesives, surfactants, emulsifiers, binders, dispersants, fertilizers etc. Boregaard

biorefinery in Norway (see Table 2) has long commercialized lignosulfonate products, and is currently dominating the market.

Lignin is presently the sole renewable source of phenolic rings, and has a great potential in the future, for producing chemical building blocks [113], which are produced currently from fossil sources; but also in applications like medicine, food [114], materials, etc. A small list of proposed products are illustrated in Figure 7.

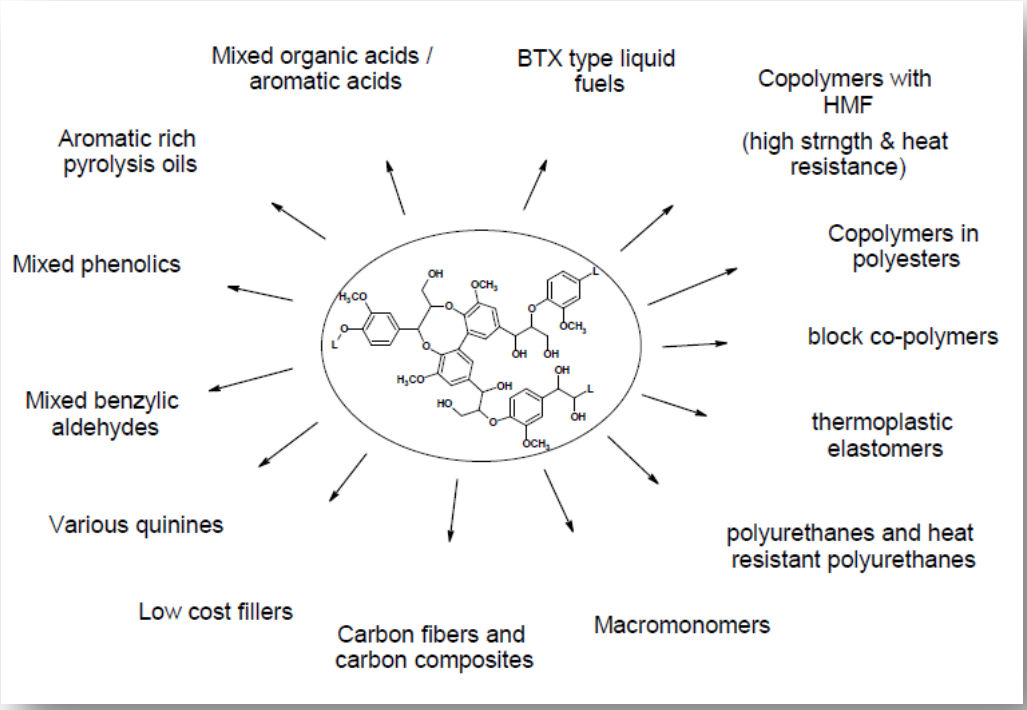

Figure 7. New product opportunities from lignin [5]

In this oilseed rape biorefinery lignin is the solid residue of ethanol production line. Rape straw lignin in this case can be burned in a CHP plant [62] attached to the biorefinery for production of electricity and steam for the biorefinery. Lignin has LHV similar to straw and is an excellent fuel due to its lower ash content (1.8%) than rape straw (5%). This technology is mature and there is already a created market for straw lignin from cellulosic ethanol biorefineries.

Alternatively, gasification of rape straw lignin for producing syngas is considered. Gasification with supercritical water [115] is a particularly economic method for wet lignin [116]. Syngas can be either burned locally for feeding onsite energy needs, or cleaned to hydrogen gas and used as chemical intermediate. Technology of gasification of biomass has been commercialized by Chemrec in Sweden that produces DME (dimethyl ether) from waste streams of pulp and paper industry via gasification and FT technology. Also, Chrisgas in Sweden has a pilot plant that investigates the production of hydrogen gas from wood chips and straw pellets. Gasification technology of lignin represents a realistic alternative to combustion for 2020, and has the flexibility of using the product (syngas) as fuel or building block. FT technology for methanol and DME is taking off presently, and by 2020 is going to be mature.

2.5 Biogas from all the waste streams of the oilseed rape biorefinery

The studied biorefinery contains three major wastewater steams: 1) wastewater from biodiesel production line. In the case biodiesel was produced by lipases, wastewater sources exclusively [38] from washing facilities (oil crushing, biodiesel reactors, piping etc [12]). However, for this type of wash water, data were not found in literature. In the case, biodiesel is produced by mineral soluble catalyst the relative volume of wash water is high and composition is presented in Table 9; 2) thin stillage from cellulosic ethanol line [117-120]. After ethanol distillation, thin stillage contains C5 sugars, short-chain cells, enzymes, organic acids, phenols, and furans. Composition of wheat straw stillage is presented in Table 9; and 3) residual liquid fraction from pretreatment of rape straw [75] that was not used for enzyme production. Liquid fraction contains mainly C5 sugars short-chain organic acids, and some microbial inhibitors, furans, and phenols. Composition of liquid fraction of rape straw is presented in Table 9. Potential

wastes from processing/refining of glycerol, cellulases, or rapeseed cake are not considered here.

Table 9 Composition of three major wastewater streams of the biorefinery. Biodiesel effluent is based on KOH catalysts [6]. Stillage composition as an example, is derived from wheat straw [13]

	Hydrolysate (unpublished data)	Stillage	Biodiesel washwater
pH	3.9	3.6	5.5-9
COD (g/l)	NA	150	312-588
Glucose (g/l)	1.03	0.1	NA
Xylose (g/l)	6.65	8.2	NA
VFA (g/l)	1.82	0.18	NA
Acetic acid (g/l)	0.91	NA	NA
Ethanol (g/l)	ND	2.3	NA
Furfural (g/l)	0.08	ND	NA
HMF (g/l)	0.01	ND	NA
Phenolics (g/l)	1.34	0.06	NA
Oil and grease (g/l)	ND	ND	18-22
Total nitrogen (TKN g/l)	0.2	1.4	0.44-0.46

NA= Not analyzed,ND= Not detected

Most valuable component in the presented lignocellulosic waste streams is the C5 sugars. C5 sugars have already been suggested for production of cellulases. Regarding, further exploitation of C5 sugars, several R&D projects are connecting C5 sugars with production of chemicals (furan derivatives, sweeteners), fuels (ethanol, biogas and hydrogen) and materials (biocomposites), but all are at early stage development. A remarkable potential product from C5 sugars is xylitol. Xylitol is a sweetener in food industry, produced by catalytic hydrogenation of xylose from hardwood, fruits or corn. So far, xylitol fermentation from lignocellulosic C5 sugars have not managed to compete chemical production methods of xylitol. Alternatively wastewater streams of the biorefinery can be used for biogas production.

Anaerobic digestion (AD) is a mature scaled-up technology exploited all over the world. In AD, mixed bacterial culture degrades virtually any type of organic matter into mainly CH_4 and CO_2 (biogas). In addition, the effluent from AD contains nutrients that can be used as fertilizer back to the field for production of oilseed rape. According to EurObservEr, 5.9 MiToe of biogas were produced in Europe in 2007 derived from landfill (49.2%), wastewater treatment (15%), and other sources (35.7%) [89]. Denmark is the 4th major biogas producer per capita (18 Toe/1000 inhabitants), and in 2007 Denmark had 160 AD units. Biogas fuel has many applications, like production of heat (19-26 MJ/m^3), cogeneration of heat and electricity (in CHP), and upgrading to natural gas or fuel gas quality [121].

Biodegradable organic load of the waste streams presented in Table 9 make it an ideal substrate for anaerobic digestion for biogas production. Moreover, mixing the above wastewater streams is beneficial for biogas production [122]. Methane content in biogas depends on substrate, and oil particularly gives a high methane content [122]. The oleaginous wastewater contains very low nitrogen content that inhibit bacteria in AD, therefore it needs to be codigested [123,124] or blended with nutrients [32] for increase of nitrogen source, and diluted [32]. The nitrogen source and dilution can be provided by the other two effluent streams (Table 9).

The stillage from production of sugar-, starch- ethanol, and cellulosic-ethanol have many similarities, and this is useful here, since data on AD of lignocellulose ethanol stillage are very limited in literature [13,117,125,126]. Wilkie et al. (2000) reviewed that AD of stillage from sugar and starch ethanol at thermophilic conditions has double productivities compared to mesophilic conditions for the same quality of effluent [119]. Thermophilic conditions are suitable for lignocellulosic effluents since they have already an increased temperature (from distillation, pretreatment). Kaparaju et al. (2010) reported that stillage from wheat straw after diluted by 75% was digested at thermophilic conditions, and 76% of COD (Chemical Oxygen Demand) was removed at Organic Loading Rate (OLR) of 17.1 g COD l^{-1} d^{-1}) and hydraulic retention time (HRT) of 20 days [117]. However, Uellendahl and Ahring (2010) showed that mesophilic conditions can support higher OLR than 8.8 gCOD l^{-1}

d^{-1} at a UASB reactors treating lignocellulosic bioethanol stillage without dilution [126]. Finally Luo et al. presented an oilseed rape biorefinery in which all waste streams (stillage, residual liquid fraction from pretreatment, glycerol, rape seed cake) were processed by AD for biogas production, and achieved 80-90% organic load degradability, and product yields (310-400 ml CH_4/ gram volatile solids.

AD is an important part of lignocellulosic ethanol biorefineries and has been proposed [103] and planned to couple cellulosic ethanol projects in demo-scale and full scale biorefineries, like Inbicon and BornBiofuel in Denmark, or Poet in USA (Liberty project). Currently, there is no other competitive solution for treating the mixture of wastes with coproduction of energy at industrial scale [90], although of the stream of C5 sugars exiting the conventional ethanol production can be source for several added value products. In this biorefinery determination of composition and volumes of effluents is required for deciding on the exact setup of AD (thermophilic, UASB reactor system). However, this technology is mature enough and has been tested with similar effluents, so that it can be realistically included to this biorefinery for scaling up for 2020.

2.6 Sustainability considerations for biorefineries

When a biorefinery is designed many parameter are involved in the environmental sustainability background of the project. Common environmental indicators of LCA analysis are water consumption, (eco)toxicity, acidification, eutrophication etc [127]. Except these indicators, other energy and other environmental sustainability parameters need to be taken into account in a biofuel biorefinery, like:

- The Energy Return On Investment (EROI) is the ratio of the energy that returns to society over the energy that is removed from society by an activity [128]. EROI is crucial for assessing the net displacement of fossil fuels by use of biomass for production of biofuels.

- Carbon intensity is used for measuring the mitigation of GHG emissions by substituting fossil fuels.
- Finally, Indirect Land Use Change (ILUC) is assessed when land is used for production of energy, chemicals and materials in addition to food, feed.

Energywise, growing oilseed rape requires (fossil) energy for tillage, chemical production and application on land (fertilizers, pesticides), machinery production, harvesting, crop transport and storage, and labor. But also processing of oilseed rape requires pumps, presses, heaters, mixers, vessels, chemical production (catalysts), energy for running the whole biorefinery and labor. According to Cherubini and Stromman [129] by processing biomass into biofuels, the energy bound to biomass is concentrated into energy carriers (fuels) primarily by removing oxygen (in form of CO_2) or adding hydrogen (in form of water). However, by adding processes to the process design of the biorefinery energy is spent for running it. Finally, biofuel products require energy and labor to reach end-users. If straw is not ploughed back to the land, then it requires energy and labor for baling, transport, storage [54], and finally processing. Big steps towards higher energy efficiency of biorefineries and automotive engines is expected in the future. However, such a potential is not expected in production of biomass.

Calculation of EROI of rapeseed biodiesel is not an easy task, and is determined by the boundaries of the system, the geographical location, the size, and the allocation of co-products to markets. Thamsiriroj and Murphy (2010) [54] calculated that for a rapeseed biodiesel plant without co-product allocation, the net energy produced was 23 GJ/y per hectare. This base scenario resulted in production of 47.74 kg CO_2/GJ biodiesel GHG emissions (that is equal to 4.4% reduce of GHG emission compared to diesel). But, when rapeseed cake and rape straw were used for certain markets, the picture greatly improved. Particularly, use of rapeseed cake as cattle feed supplement reduced net GHG emissions of biodiesel biorefinery to -21.1 kg CO_2/GJ biodiesel GHG emissions (105% decrease compare to diesel), and when rape straw was burned in CHP plant, the base scenario of GHG emissions decreased to -51.9 kg CO_2/GJ biodiesel (135%). Comparing also net

energy production for the last two scenarios were 102.9 GJ/y per hectare and 113.7 GJ/y per hectare respectively [54]. This study clarifies the need to allocate industrial residues to a market, for reducing the implication of the required energy and material intensive agricultural activities, and also replace products that need extra land use to be produced (like soy beans for fodder). Therefore increase efficiency of land use.

Von Blottnitz has reviewed 47 LCA-based studies and concluded that ethanol production from lignocellulosic residues results in 25-90 GJ/y per hectare net energy production, although land is not dedicated for production of this biomass. In the same study, by far the most efficient energy crop was sugarcane in Brazil and sugar beet in UK with net energy 250 GJ/y and 120 GJ/y per hectare of land cropped [130]. The EROI for cellulosic ethanol for wheat straw grown in UK was 5.2.

In parallel to EROI, carbon efficiency is a crucial environmental factor, especially regarding short life-span products like biofuels. Both fossil fuels and biofuels contain variable amount of carbon weight per energy content (Coal 97 kg CO_2/GJ; Oil 69 kg CO_2/GJ; natural gas 54 kg CO_2/GJ; biomass 86 kg CO_2/GJ; diesel 83 kg CO_2/GJ; REE biodiesel 48 kg CO_2/GJ [50]). Again in the review of von Blottnitz, lignocellulosic ethanol had a potential of 2,000-6,000 kg CO_2/y per hectare.

Land use change is not considered in this biorefinery scenario. This study assumes that rapeseed biodiesel is already an established biodiesel production industry in Europe with established agricultural production systems. However, the development of a whole crop oilseed biorefinery biorefinery aims to increase efficiency of land use, by exploitation of biodiesel residues for production of bioethanol, biogas, and electricity and heat.

For this biorefinery LCA analysis has not been done yet, but is essential for having a bright picture of the net profit of developing such a complex biorefinery for industrial residues of rapeseed biodiesel by 2020.

2.7 Discussion

In this chapter, a case study was analyzed of how a contemporary “One platform (oil) biorefinery for biodiesel, glycerol, and animal feed, from rapeseed” can be upgraded to a “Four platforms (C6 sugars, biogas, lignin, and oil) biorefinery for biodiesel, chemical building blocks, ethanol, electricity and heat from oilseed rape” by 2020 in Europe.

In this scenario 1) production method of rapeseed biodiesel was upgraded, according to latest findings in pilot/demo studies, and 2) technologies for processing biodiesel residues were integrated, shaping a whole-crop biorefinery from oilseed rape. The products were selected according to a list of criteria. The target was to give suggestions for a realistic biorefinery project of demo scale by 2020 of Europe. In literature, reviews were found for exploiting only the potential uses of rapeseed cake [44,131]. In this study, whole-crop is utilized, including rapeseed cake, rape straw and crude glycerol.

The rape whole crop biorefinery of this scenario is presented as a whole in Figure 8. The biorefinery is divided into two major branches; the rapeseed line that included the oil platform, and the lignocellulosic line that included 3 platforms C6 sugars, mbiogas, and lignin. The straw and the rapeseed are separated in the field and transported to the biorefinery. In rapeseed line, the rapeseed is separated into rapeseed oil and rapeseed cake. The rapeseed oil is processed to biodiesel and crude glycerol. Glycerol can processed into intermediate chemicals like methanol. Methanol can be recycled back to biodiesel plant and used as reagent. The rapeseed cake preferably of low fiber variety (oilseed rape triple low variety) is assessed as very nutritious and efficient animal fodder, although use as culture medium for internal uses is promising.

Rape straw is separated by wet oxidation, and into a cellulose and lignin soild fraction remain whereas and a water soluble C5 sugars fraction. Cellulose is prioritized to be processed into ethanol via enzyme hydrolysis and ethanol fermentation, while hemicellulose is used for enzyme and biogas production. Anaerobic digestion utilizes C5 sugars combined with thin stillage, and the

biodiesel washing water. Lignin and biogas are burned in a CHP plant as an exchange for the energy and steam demands of the biorefinery. Figure 8 summarizes also the mass and energy balances per hectare of land cropped, based on stoichiometric and acquired results.

In Figure 8 internal symbiosis in the biorefinery highlights the benefits in energy and material sustainability. For example, ethanol is partially recycled to produce ethanol biodiesel (25% wt). Alternatively, glycerol is proposed to be used for production of methanol for producing methanol biodiesel (replacing rape straw ethanol); this scenario would save for the market 0.21 T/ha of ethanol (this scenario is not shown in Figure 8). Also cellulolytic enzymes are produced by using 20% of cellulose available in pretreated rape straw, and recycled back to hydrolyze the remaining 80% of cellulose for ethanol production. Finally, in principle when biogas and lignin are burned in CHP can cover in excess steam and electricity needs of the biorefinery [62].

Some ideas that were not analyzed in previous paragraphs, for producing sophisticated products (HVLV) from oilseed rape, instead of the above studied super-commodity biofuels (LVHV), are proposed:

1. Except ethanol, numerous products can be produced from rape straw sugars that are presently produced from sugars or starch (see Table 2), like fibers, plastics (like PLA), textiles (poly esters), chemicals (furanics), fuels (FDCA), soaps, pharmaceuticals, cosmetics, food ingredients (cellodextrines), enzymes (protease, hemicellulases), pyrolytic oil, and more. The technologies are inherited from starch and sugar industry, but need process adaptation.
2. Glucosinolates are excellent natural herbicides present in rapeseed cake, but their extraction and valorization needs further research [132]. In this study, glucosinolates were not exploited, due to selection of cropping variety with low glucosinolate content (triple low variety), giving priority to the use of rapeseed cake as animal fodder. However, there is a big potential coming from exploitation of wild type rapeseed that is cultivated in Europe for other uses.

3. Lipases can be produced from waste rapeseed oil and rapeseed cake, in similar fashion to production of cellulases. Such attempts have been recorded in literature [133].
4. As discussed for the rape straw lignin, also in the hulls of brown rapeseed cake, there are phenolic compounds and tannins that are excellent sources of phenolic derivatives and are currently are produced from fossil oil [134-136].

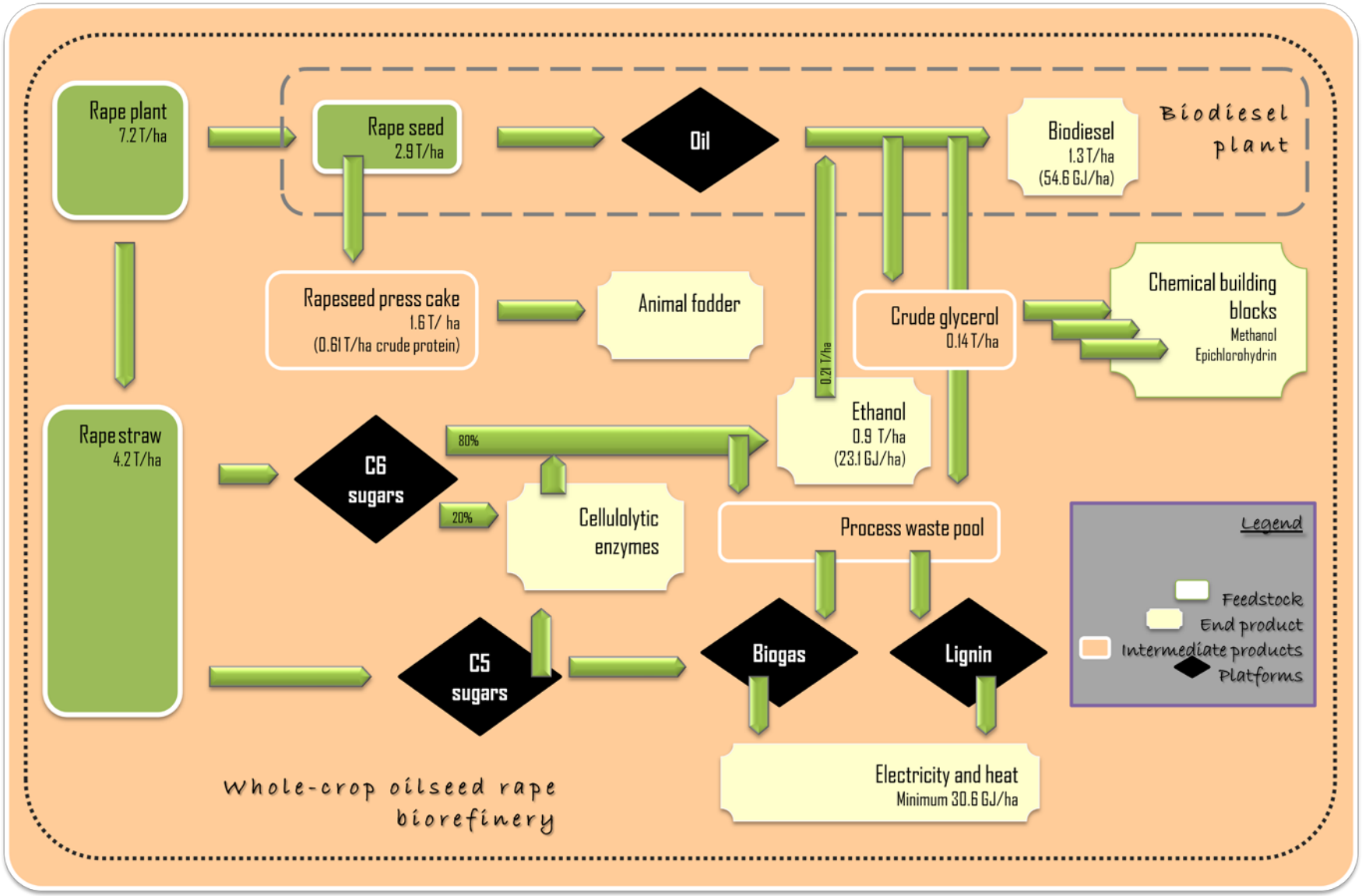

Figure 8 The product line of the rapeseed biodiesel plant and the expanded whole-crop oilseed rape biorefinery

2.8 Future Outlook

Time for implementing such the oilseed biorefinery project is selected based on the necessity for the suggested processing technologies to be refined, tested at high scale, and mature by 2020, and become economically attractive and competitive against petro-refineries [11]. Examples of processes that expect to face cost reduction in the next 10 years, are pretreatment methods of rape straw, downstream processes like for bioethanol production [137], and costs of enzyme biocatalysts like lipases [38]. This biorefinery needs systematic economic analysis, in addition to LCA analysis. Final selection of technologies and products will be drawn from the modeling techno-economic analysis hand by hand with process optimization studies.

Especially the setup of ethanol production setup beyond SSF is expected to face major changes in the future. A new technological setup for facilitating enzyme hydrolysis and fermentation of C6 and/or C5 sugars to ethanol needs to be designed, since current system is inefficient working at sub-optimal conditions for biocatalysts. The new setup will be designed based on the capacities of the biocatalysts (enzymes, yeasts, bacteria). New efficient biocatalysts also will be produced that will have novel promising traits, like C6/C5 sugar fermentation, thermotollerance, enzyme secretion etc.

Presently, biorefineries that are based on biomass processing are the only solution towards production of biofuels and bioproducts. In the future, materials, chemicals, and fuels can also be produced with capturing and fixing CO_2 by electrochemical synthesis methods, using directly the energy from the sun (artificial photosynthesis), wind, geothermal energy or other renewable sources of electricity with catalysts [138] or biocatalysts [139]. In July 2010, USA DOE granted $122M for a research unit for developing biofuels by artificial photosynthesis from the sun, without use of plants. Alternatively to carbon capture storage, artificial photosynthesis has a tremendous potential to fix carbon from flue gases into utile products.

Nowadays, complex biorefineries are facing major economical challenges with fresh scaled up technologies, while making huge

capital investments. For this, industry needs incentives and economical support for making new investments, including secured loans, and long pay back periods. Exploiting all residues for added-value products is the first step to increase revenues in the biorefiney. For instance, economics of cellulosic ethanol production are very negative if residues are not valorized [140]; exploitation of side-products like hemicellulose and lignin [18,118,141] secures revenues [87]. More than that, portfolio of products in biorefineries needs to be supplemented by HVLV chemicals. Nowadays, the R&D is making small but steady steps towards production of chemicals from biomass residues like in the case of glycerol. Special policies, directives, mandates, and subsidies need to be deployed by EU to move the market towards production of biobased chemicals in a biorefinery concept. So that, biorefinery industry will succeed wood biorefineries have shown for more than half a century.

The opportunities for developing biobased products are unlimited and development of biorefineries is based on the infrastructural and economical capacities and needs. At this point, R&D needs data from scaled up projects to help improve processibility of biomass, therefore close collaboration from industry and academia is a win-win solution.

Reference List

[1] Canola meal: feed industry guide. Newkirk R, editor. 2009;4. Canada, Canola Council. 6-12-2010.
Ref Type: Report

[2] Gamba L. Sustainable biofuels and the role of cellulosic ethanol. 13-10-2010. 28-10-2010.
Ref Type: Slide

[3] Bell JM. Factors Affecting the Nutritional-Value of Canola-Meal - A Review. Canadian Journal of Animal Science 1993; 73(4):679-97.

[4] Bioconversion of crop residues and wastes into ethanol, with emphasis on chemical and enzymic hydrolysis of fibres. Wageningen UR-Food & Biobased Research; 2007.

[5] Hollaway JE, Bozell JJ, White JF, johnson D. Top value-added chemicals from biomass: Volume II - Results for potentail candidated from biorefinery lignin. 1-10-2007. Pacific Northwest National Laboratory, US, US. Department of Energy. 6-12-2010.
Ref Type: Report

[6] Jaruwat P, Kongjao S, Hunsom M. Management of biodiesel wastewater by the combined processes of chemical recovery and electrochemical treatment. Energy Conversion and Management 2010; 51(3):531-7.

[7] Bozell JJ, Petersen GR. Technology development for the production of biobased products from biorefinery carbohydrates-the US Department of Energy's "Top 10" revisited. Green Chemistry 2010; 12(4):539-54.

[8] Alexander BW, Gordon AH, Lomax JA, Chesson A. Composition and Rumen Degradability of Straw from 3 Varieties of Oilseed Rape Before and After Alkali, Hydrothermal and Oxidative Treatment. Journal of the Science of Food and Agriculture 1987; 41(1):1-15.

[9] Rashid U, Anwar F. Production of biodiesel through optimized alkaline-catalyzed transesterification of rapeseed oil. Fuel 2008; 87(3):265-73.

[10] Moreira ABR, Perez VH, Zanin GM, de Castro HF. Biodiesel synthesis by enzymatic transesterification of palm oil with ethanol using lipases from several sources immobilized on silica-PVA composite. Energy & Fuels 2007; 21(6):3689-94.

[11] Smith W. Mapping the development of UK Biorefinery Complexes. 2007. Tamutech Consuntancy, National Non Food Crops Centre. 7-9-2010.
Ref Type: Report

[12] Leung DYC, Wu X, Leung MKH. A review on biodiesel production using catalyzed transesterification. Applied Energy 2010; 87(4):1083-95.

[13] Kaparaju P, Serrano M, Thomsen AB, Kongjan P, Angelidaki I. Bioethanol, biohydrogen and biogas production from wheat straw in a biorefinery concept. Bioresource Technology 2009; 100(9):2562-8.

[14] Sun H, Hu K, Lou H, Zheng XM. Biodiesel production from transesterification of rapeseed oil using KF/Eu2O3 as a catalyst. Energy & Fuels 2008; 22(4):2756-60.

[15] Encinar JM, Gonzalez JF, Rodriguez JJ, Tejedor A. Biodiesel fuels from vegetable oils: Transesterification of Cynara cardunculus L. oils with ethanol. Energy & Fuels 2002; 16(2):443-50.

[16] Cherubini F, Jungmeier G, Wellisch M, Willke T, Skiadas I, Van Ree R et al. Toward a common classification approach for biorefinery systems. Biofuels Bioproducts & Biorefining-Biofpr 2009; 3(5):534-46.

[17] Saka S, Kusdiana D. Biodiesel fuel from rapeseed oil as prepared in supercritical methanol. Fuel 2001; 80(2):225-31.

[18] Lyko H, Deerberg G, Weidner E. Coupled production in biorefineries-Combined use of biomass as a source of energy, fuels and materials. Journal of Biotechnology 2009; 142(1):78-86.

[19] Li LL, Du W, Liu DH, Wang L, Li ZB. Lipase-catalyzed transesterification of rapeseed oils for biodiesel production with a novel organic solvent as the reaction medium. Journal of Molecular Catalysis B-Enzymatic 2006; 43(1-4):58-62.

[20] Wittkop B, Snowdon RJ, Friedt W. Status and perspectives of breeding for enhanced yield and quality of oilseed crops for Europe. Euphytica 2009; 170(1-2):131-40.

[21] Karaosmanoglu F, Tetik E, Gurboy B, Sanli I. Characterization of the straw stalk of the rapeseed plant as a biomass energy source. Energy Sources 1999; 21(9):801-10.

[22] Energy and greenhouse gas balances of biofuels' production chains in France - Executive summary. france: ADEME; 2002.

[23] Unger E. Commercial processing of canola and rapeseed: Crushing and oil extraction. Canola and Rapeseed: Production, Chemistry, Nutrition, and Processing Technology, New York: Van Nostrand Reinhold; 1990, p. 235-249.

[24] Nag A. Processing vegetable oils as biodiesel and engine performance. In: Nag A, editor. Biofuels refining and performance, 1st. ed. Ney York: McGraw-Hill; 2008, p. 165-190.

[25] Pinto AC, Guarieiro LLN, Rezende MJC, Ribeiro NM, Torres EA, Lopes WA et al. Biodiesel: An overview. Journal of the Brazilian Chemical Society 2005; 16(6B):1313-30.

[26] European Biodiesel Board. 2010. 20-9-2010.
Ref Type: Internet Communication

[27] Larsen R. Rapeseed varieties and state of the art of rapeseed cultivations in Europe. 2010. 8-11-2010.
Ref Type: Personal Communication

[28] Marchetti JM, Miguel VU, Errazu AF. Possible methods for biodiesel production. Renewable & Sustainable Energy Reviews 2007; 11(6):1300-11.

[29] Jackson MA, King JW. Methanolysis of seed oils in flowing supercritical carbon dioxide. Journal of the American Oil Chemists Society 1996; 73(3):353-6.

[30] Helwani Z, Othman MR, Aziz N, Fernando WJN, Kim J. Technologies for production of biodiesel focusing on green catalytic techniques: A review. Fuel Processing Technology 2009; 90(12):1502-14.

[31] GHP-Biodiesel. Biodiesel Installation GHP Module 25K. 2008. Neumarkt, Germany. 27-9-2010.
Ref Type: Report

[32] Suehara K, Kawamoto Y, Fujii E, Kohda J, Nakano Y, Yano T. Biological treatment of wastewater discharged from biodiesel fuel production plant with alkali-catalyzed transesterification. Journal of Bioscience and Bioengineering 2005; 100(4):437-42.

[33] Axens IFP Group technologies. Biodiesel: Diester Industrie Selects Axens' Renewable Energy Technology. Axens website, press room 2004.

[34] Tan TW, Lu JK, Nie KL, Deng L, Wang F. Biodiesel production with immobilized lipase: A review. Biotechnology Advances 2010; 28(5):628-34.

[35] Ma FR, Clements LD, Hanna MA. Biodiesel fuel from animal fat. Ancillary studies on transesterification of beef tallow. Industrial & Engineering Chemistry Research 1998; 37(9):3768-71.

[36] Warabi Y, Kusdiana D, Saka S. Reactivity of triglycerides and fatty acids of rapeseed oil in supercritical alcohols. Bioresource Technology 2004; 91(3):283-7.

[37] Fjerbaek L, Christensen KV, Norddahl B. A Review of the Current State of Biodiesel Production Using Enzymatic Transesterification. Biotechnology and Bioengineering 2009; 102(5):1298-315.

[38] Sotoft LF, Rong BG, Christensen KV, Norddahl B. Process simulation and economical evaluation of enzymatic biodiesel production plant. Bioresource Technology 2010; 101(14):5266-74.

[39] Ma FR, Hanna MA. Biodiesel production: a review. Bioresource Technology 1999; 70(1):1-15.

[40] Granda CB, Zhu L, Holtzapple MT. Sustainable liquid biofuels and their environmental impact. Environmental Progress 2007; 26(3):233-50.

[41] Johnson DT, Taconi KA. The glycerin glut: Options for the value-added conversion of crude glycerol resulting from biodiesel production. Environmental Progress 2007; 26(4):338-48.

[42] Volume I: Results from screening for potential candidates fom sugars and synthesis gas. Werpy T, Petersen GR, editors. 2004;1. US. DoE, Biomass. Top value chemicals from Biomass.
Ref Type: Report

[43] Pagliaro M, Ciriminna R, Kimura H, Rossi M, la Pina C. From glycerol to value-added products. Angewandte Chemie-International Edition 2007; 46(24):4434-40.

[44] Koutinas AA, Wang RH, Webb C. The biochemurgist - Bioconversion of agricultural raw materials for chemical production. Biofuels Bioproducts & Biorefining-Biofpr 2007; 1(1):24-38.

[45] Dasari MA, Kiatsimkul PP, Sutterlin WR, Suppes GJ. Low-pressure hydrogenolysis of glycerol to propylene glycol. Applied Catalysis A-General 2005; 281(1-2):225-31.

[46] U.S.EPA GC. 2006 Academic Award: Professor Galen J. Suppes, University of Missouri-Columbia for "Biobased Propylene Glycol and Monomers from Natural Glycerin". 2006. 30-9-2010.
Ref Type: Internet Communication

[47] PharmaTech.com. ADM Plans Green Production for USP-Grade Propylene Glycol. 2009. 30-9-2010.
Ref Type: Internet Communication

[48] EPICEROL®: An innovative environmental breakthrough in Epichlorohydrin production. Chemicals-Technology.com 23-9-2010. 1-10-2010.
Ref Type: Internet Communication

[49] The BIO-MCN production process. 2010. 1-10-2010.
Ref Type: Internet Communication

[50] Peterson CL, Hustrulid T. Carbon cycle for rapeseed oil biodiesel fuels. Biomass & Bioenergy 1998; 14(2):91-101.

[51] Canola meal: feed industry guide. Newkirk R, editor. 2009;4. Canada, Canadian International Grains Insitute. 10-12-2010.
Ref Type: Report

[52] Siddiqui IR, Wood PJ. Carbohydrates of Rapeseed - Review. Journal of the Science of Food and Agriculture 1977; 28(6):530-8.

[53] van Gelder J.W., Kammeraat K, Kroes H. Soy consumption for feed and fuel in the European Union. 28-10-2008;Final. Netherlands, Friends of the Earth. 17-11-2010.
Ref Type: Report

[54] Thamsiriroj T, Murphy JD. Can Rape Seed Biodiesel Meet the European Union Sustainability Criteria for Biofuels? Energy & Fuels 2010; 24:1720-30.

[55] Weremko D, Fandrejewski H, Zebrowska T, Han IK, Kim JH, Cho WT. Bioavailability of phosphorus in feeds of plant origin for pigs. Asian-Australasian Journal of Animal Sciences 1997; 10(6):551-66.

[56] Ramachandran S, Singh SK, Larroche C, Soccol CR, Pandey A. Oil cakes and their biotechnological applications - A review. Bioresource Technology 2007; 98(10):2000-9.

[57] Wang RH, Shaarani SM, Godoy LC, Melikoglu M, Vergara CS, Koutinas A et al. Bioconversion of rapeseed meal for the production of a generic microbial feedstock. Enzyme and Microbial Technology 2010; 47(3):77-83.

[58] Yang B, Wyman CE. Pretreatment: the key to unlocking low-cost cellulosic ethanol. Biofuels Bioproducts & Biorefining-Biofpr 2008; 2(1):26-40.

[59] Chang VS, Holtzapple MT. Fundamental factors affecting biomass enzymatic reactivity. Applied Biochemistry and Biotechnology 2000; 84-6:5-37.

[60] Himmel ME. Biomass recalcitrance: engineering plants and enzymes for biofuels production (vol 315, pg 804, 2007). Science 2007; 316(5827):982.

[61] Tolan JS. Iogen's Demonstration ptocess for producing ethanol from cellulosic biomass. In: Kamm B, Gruber P, Kamm M, editors. Biorefineries - Industrial processes and products, 1st. ed. weinheim: Wiley-VCH; 2006, p. 193-208.

[62] Larsen J, Petersen MO, Thirup L, Li HW, Iversen FK. The IBUS process - Lignocellulosic bioethanol close to a commercial reality. Chemical Engineering & Technology 2008; 31(5):765-72.

[63] Galbe M, Zacchi G. Pretreatment of lignocellulosic materials for efficient bioethanol production. Biofuels 2007; 108:41-65.

[64] Carvalheiro F, Duarte LC, Girio FM. Hemicellulose biorefineries: a review on biomass pretreatments. Journal of Scientific & Industrial Research 2008; 67(11):849-64.

[65] Lu XB, Zhang YM, Angelidaki I. Optimization of H2SO4-catalyzed hydrothermal pretreatment of rapeseed straw for bioconversion to ethanol: Focusing on pretreatment at high solids content. Bioresource Technology 2009; 100(12):3048-53.

[66] Li H, Kim NJ, Jiang M, Kang JW, Chang HN. Simultaneous saccharification and fermentation of lignocellulosic residues pretreated with phosphoric acid-acetone for bioethanol production. Bioresource Technology 2009; 100(13):3245-51.

[67] Diaz MJ, Cara C, Ruiz E, Romero I, Moya M, Castro E. Hydrothermal pre-treatment of rapeseed straw. Bioresource Technology 2010; 101(7):2428-35.

[68] Targonsky Z. Autohydrolysis Extraction Process As A Pretreatment of Lignocelluloses for Their Enzymatic-Hydrolysis. Acta Biotechnologica 1985; 5(4):353-61.

[69] Sarikaya A, Ladisch MR. Solid-state fermentation of lignocellulosic plant residues from Brassica napus by Pleurotus ostreatus. Applied Biochemistry and Biotechnology 1999; 82(1):1-15.

[70] Holtzapple MT, Humphrey AE. The Effect of Organosolv Pretreatment on the Enzymatic-Hydrolysis of Poplar. Biotechnology and Bioengineering 1984; 26(7):670-6.

[71] Chum HL, Douglas LJ, Feinberg DA. Evaluation of pretreatments of biomass for enzymic hydrolysis of cellulose. 1985;1. Solar Energy Research Institute, Golden, CO, USDA. 21-9-2010. Ref Type: Report

[72] Galbe M, Sassner P, Wingren A, Zacchi G. Process engineering economics of bioethanol production. Biofuels 2007; 108:303-27.

[73] Gnansounou E, Dauriat A. Techno-economic analysis of lignocellulosic ethanol: A review. Bioresource Technology 2010; 101(13):4980-91.

[74] Kazi FK, Fortman JA, Anex RP, Hsu DD, Aden A, Dutta A et al. Techno-economic comparison of process technologies for biochemical ethanol production from corn stover. Fuel 2010; 89:S20-S28.

[75] Petersson A, Thomsen MH, Hauggaard-Nielsen H, Thomsen AB. Potential bioetanol and biogas production using lignocellulosic biomass from winter rye, oilseed rape and faba bean. Biomass & Bioenergy 2007; 31(11-12):812-9.

[76] Klinke HB, Ahring BK, Schmidt AS, Thomsen AB. Characterization of degradation products from alkaline wet oxidation of wheat straw. Bioresource Technology 2002; 82(1):15-26.

[77] Felby C, Klinke HB, Olsen HS, Thomsen AB. Ethanol from wheat straw cellulose by wet oxidation pretreatment and simultaneous saccharification and fermentation. 2003.

[78] Varga E, Schmidt AS, Reczey K, Thomsen AB. Pretreatment of corn stover using wet oxidation to enhance enzymatic digestibility. Applied Biochemistry and Biotechnology 2003; 104(1):37-50.

[79] Martin C, Klinke HB, Thomsen AB. Wet oxidation as a pretreatment method for enhancing the enzymatic convertibility of sugarcane bagasse. Enzyme and Microbial Technology 2007; 40(3):426-32.

[80] Palonen H, Thomsen AB, Tenkanen M, Schmidt AS, Viikari U. Evaluation of wet oxidation pretreatment for enzymatic hydrolysis of softwood. Applied Biochemistry and Biotechnology 2004; 117(1):1-17.

[81] Martin C, Thomsen MH, Hauggaard-Nielsen H, BelindaThomsen A. Wet oxidation pretreatment, enzymatic hydrolysis and simultaneous saccharification and fermentation of clover-ryegrass mixtures. Bioresource Technology 2008; 99(18):8777-82.

[82] Lissens G, Klinke H, Verstraete W, Ahring B, Thomsen AB. Wet oxidation treatment of organic household waste enriched with wheat straw for simultaneous saccharification and fermentation into ethanol. Environmental Technology 2004; 25(6):647-55.

[83] Frandsen RB, Montgomery M, Larsen OH. Field test corrosion experiences when co-firing straw and coal: 10 year status within Elsam. Materials at High Temperatures 2007; 24(4):343-9.

[84] Sander B. Properties of Danish biofuels and the requirements for power production. Biomass & Bioenergy 1997; 12(3):177-83.

[85] Hettenhaus J, Morris D. Feedstock supply, logistics, processing, and composition. Applied Biochemistry and Biotechnology 2004; 113:3-4.

[86] Darby RJ, Yeoman DP. Effects of Methods of Cereal Straw Disposal, Seedbed Preparation and Sowing Method on the Establishment, Yield and Oil Content of Winter Oilseed Rape (Brassica-Napus). Journal of Agricultural Science 1994; 122:393-404.

[87] Zhang YHP. Reviving the carbohydrate economy via multi-product lignocellulose biorefineries. Journal of Industrial Microbiology & Biotechnology 2008; 35(5):367-75.

[88] Global Renewable Fuels Alliance Website. http://www globalrfa org/ [2010 [cited 2010 Nov. 24]; Available from: URL:http://www.globalrfa.org/

[89] Biogas Barometer. EurObservEr [2008 [cited 2010 Oct. 11]; Available from: URL:http://www.eurobserv-er.org/pdf/baro186_a.pdf

[90] Claassen PAM, van Lier JB, Contreras AML, van Niel EWJ, Sijtsma L, Stams AJM et al. Utilisation of biomass for the supply of energy carriers. Applied Microbiology and Biotechnology 1999; 52(6):741-55.

[91] Taherzadeh MJ, Karimi K. Enzyme-Based Hydrolysis Processes for Ethanol from Lignocellulosic Materials: A Review. Bioresources 2007; 2(4):707-38.

[92] Hahn-Hagerdal B, Karhumaa K, Fonseca C, Spencer-Martins I, Gorwa-Grauslund MF. Towards industrial pentose-fermenting yeast strains. Applied Microbiology and Biotechnology 2007; 74(5):937-53.

[93] Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. Microbial cellulose utilization: Fundamentals and biotechnology. Microbiology and Molecular Biology Reviews 2002; 66(3):506-+.

[94] Otero JM, Panagiotou G, Olsson L. Fueling industrial biotechnology growth with bioethanol. 2007.

[95] Girio FM, Fonseca C, Carvalheiro F, Duarte LC, Marques S, Bogel-Lukasik R. Hemicelluloses for fuel ethanol: A review. Bioresource Technology 2010; 101(13):4775-800.

[96] Karmakar M, Ray RR. Current Trends in Research and Application of Microbial Cellulases. Research Journal of Microbiology 2011;(6):41-53.

[97] Bhat MK. Cellulases and related enzymes in biotechnology. Biotechnology Advances 2000; 18(5):355-83.

[98] Zhang YHP, Himmel ME, Mielenz JR. Outlook for cellulase improvement: Screening and selection strategies. Biotechnology Advances 2006; 24(5):452-81.

[99] Falholt P. Novozymes - Decrease project. 13-10-2010. 28-10-2010.
Ref Type: Slide

[100] Genencor Celebrates Major Progress in the Conversion of Biomass to Ethanol. Genencor website 2004.

[101] US Department of Energy (DoE) 2005.

[102] Barta Z, Kovacs K, Reczey K, Zacchi G. Process design and economics of On-site cellulase production on various carbon sources in a softwood-based ethanol plant. Enzyme research 2010.

[103] Barta Z, Reczey K, Zacchi G. Techno-economic evaluation of stillage treatment with anaerobic digestion in a softwood-to-ethanol process. Biotechnology for Biofuels 2010; 3.

[104] MacLean HL, Spatari S. The contribution of enzymes and process chemicals to the life cycle of ethanol. Environmental Research Letters 2009; 4(1).

[105] Wooley R, Ruth M, Glassner D, Sheehan J. Process design and costing of bioethanol technology: A tool for determining the status and direction of research and development. Biotechnology Progress 1999; 15(5):794-803.

[106] Olsson L, Christensen TMIE, Hansen KP, Palmqvist EA. Influence of the carbon source on production of cellulases, hemicellulases and pectinases by Trichoderma reesei Rut C-30. Enzyme and Microbial Technology 2003; 33(5):612-9.

[107] Bollok M, Reczey K. Cellulase enzyme production by various fungal strains on different carbon sources. Acta Alimentaria 2000; 29(2):155-68.

[108] Juhasz T, Szengyel Z, Reczey K, Siika-Aho M, Viikari L. Characterization of cellulases and hemicellulases produced by Trichoderma reesei on various carbon sources. Process Biochemistry 2005; 40(11):3519-25.

[109] Mohagheghi A, Grohmann K, Wyman CE. Production of Cellulase on Mixtures of Xylose and Cellulose. Applied Biochemistry and Biotechnology 1988; 17:263-77.

[110] Palmqvist E, HahnHagerdal B, Szengyel Z, Zacchi G, Reczey K. Simultaneous detoxification and enzyme production of hemicellulose hydrolysates obtained after steam pretreatment. Enzyme and Microbial Technology 1997; 20(4):286-93.

[111] Thygesen A, Thomsen AB, Schmidt AS, Jorgensen H, Ahring BK, Olsson L. Production of cellulose and hemicellulose-degrading enzymes by filamentous fungi cultivated on wet-oxidised wheat straw. Enzyme and Microbial Technology 2003; 32(5):606-15.

[112] Zhang SP, Marechal F, Gassner M, Perin-Levasseur Z, Qi W, Ren ZW et al. Process Modeling and Integration of Fuel Ethanol Production from Lignocellulosic Biomass Based on Double Acid Hydrolysis. Energy & Fuels 2009; 23:1759-65.

[113] Hollaway JE, Bozell JJ, White JF, johnson D. Top value-added chemicals from biomass: Volume II - Results for potentail candidated from biorefinery lignin. 1-10-2007. Pacific Northwest National Laboratory, US, US. Department of Energy. 6-12-2010.
Ref Type: Report

[114] Hollaway JE, Bozell JJ, White JF, johnson D. Top value-added chemicals from biomass: Volume II - Results for potentail candidated from biorefinery lignin. 1-10-2007. Pacific Northwest National Laboratory, US, US. Department of Energy. 6-12-2010.
Ref Type: Report

[115] Resende FLP, Fraley SA, Berger MJ, Savage PE. Noncatalytic gasification of lignin in supercritical water. Energy & Fuels 2008; 22(2):1328-34.

[116] Briens C, Piskorz J, Berruti F. Biomass valorization for fuel and chemicals production - A review. International Journal of Chemical Reactor Engineering 2008; 6.

[117] Kaparaju P, Serrano M, Angelidaki I. Optimization of biogas production from wheat straw stillage in UASB reactor. Applied Energy 2010; 87(12):3779-83.

[118] Ahring BK, Westermann P. Coproduction of bioethanol with other biofuels. Biofuels 2007; 108:289-302.

[119] Wilkie AC, Riedesel KJ, Owens JM. Stillage characterization and anaerobic treatment of ethanol stillage from conventional and cellulosic feedstocks. Biomass & Bioenergy 2000; 19(2):63-102.

[120] Exergetic analysis of anaerobic digestion of stillage: Case study in Brazil. International Conference on Efficiency, Cost Optimisation, Simulation and Environmental Aspects of Energy and Process Systems; 10 Nov 23; TWENTE ENSCHEDE, NETHERLANDS: 2000.

[121] de Mes TZD, Stams AJM, Reith JH, Zeeman G. Methane production by anaerobic digestion of wastewater and soliid wastes. In: Reith JH, Wijffels RH, Barten H, editors. Bio-methane & Bio-hydrogen, Petten: Dutch Biological Hydrogen Foundation, Energy Research Centre of the Netherlands ECN, Unit biomass; 2003, p. 58-102.

[122] Drapcho CM, Nhuan PN, Walker TH, Henson JM. Methane. Biofuels Engineering Process Technology, Ney York: McGraw-Hill; 2010, p. 329-345.

[123] Siles JA, Martin MA, Chica AF, Martin A. Anaerobic co-digestion of glycerol and wastewater derived from biodiesel manufacturing. Bioresource Technology 2010; 101(16):6315-21.

[124] Nakashimada Y, Kikuzaki T, Nishio N. Hydrogen and ethanol production from bio-diesel waste containing high concentration of glycerol using newly isolated Enterobacter aerogenes HU201. Journal of Bioscience and Bioengineering 2009; 108:S117-S118.

[125] Torry-Smith M, Sommer P, Ahring BK. Purification of bioethanol effluent in an UASB reactor system with simultaneous biogas formation. Biotechnology and Bioengineering 2003; 84(1):7-12.

[126] Uellendahl H, Ahring BK. Anaerobic Digestion as Final Step of a Cellulosic Ethanol Biorefinery: Biogas Production From Fermentation Effluent in a UASB Reactor-Pilot-Scale Results. Biotechnology and Bioengineering 2010; 107(1):59-64.

[127] Lechon Y, de la Rua C, Saez R. Life cycle environmental impacts of electricity production by solarthermal power plants in Spain. Journal of Solar Energy Engineering-Transactions of the Asme 2008; 130(2).

[128] Giampietro M, Mayumi K. A reality check on the feasibility and desirability of Agro-biofuels. The Biofuel Delusion: The fallacy of large-scale agro-biofuels production, Earthscan; 2009.

[129] Cherubini F, Stromman AH. Production of Biofuels and Biochemicals from Lignocellulosic Biomass: Estimation of Maximum Theoretical Yields and Efficiencies Using Matrix Algebra. Energy & Fuels 2010; 24:2657-66.

[130] von Blottnitz H, Curran MA. A review of assessments conducted on bio-ethanol as a transportation fuel from a net energy, greenhouse gas, and environmental life cycle perspective. Journal of Cleaner Production 2007; 15(7):607-19.

[131] Zabaniotou A, Ioannidou O, Skoulou V. Rapeseed residues utilization for energy and 2nd generation biofuels. Fuel 2008; 87(8-9):1492-502.

[132] Bjorkqvist B, Hase A. Separation and Determination of Intact Glucosinolates in Rapeseed by High-Performance Liquid-Chromatography. Journal of Chromatography 1988; 435(3):501-7.

[133] Li N, Zong MH. Lipases from the genus Penicillium: Production, purification, characterization and applications. Journal of Molecular Catalysis B-Enzymatic 2010; 66(1-2):43-54.

[134] Garrote G, Cruz JM, Moure A, Dominguez H, Parajo JC. Antioxidant activity of byproducts from the hydrolytic processing of selected lignocellulosic materials. Trends in Food Science & Technology 2004; 15(3-4):191-200.

[135] Naczk M, Amarowicz R, Shahidi F. Canola/rapeseed hull phenolics as potential free radical scavengers. 2001.

[136] Naczk M, Pegg RB, Zadernowski R, Shahidi F. Radical scavenging activity of canola hull phenolics. Journal of the American Oil Chemists Society 2005; 82(4):255-60.

[137] Vane LM. A review of pervaporation for product recovery from biomass fermentation processes. Journal of Chemical Technology and Biotechnology 2005; 80(6):603-29.

[138] Sequeira CAC, Santos DMF. Electrochemical Routes for Industrial Synthesis. Journal of the Brazilian Chemical Society 2009; 20(3):387-406.

[139] Rabaey K, Rozendal RA. Microbial electrosynthesis - revisiting the electrical route for microbial production. Nature Reviews Microbiology 2010; 8(10):706-16.

[140] Wingren A, Galbe M, Zacchi G. Techno-economic evaluation of producing ethanol from softwood: Comparison of SSF and SHF and identification of bottlenecks. Biotechnology Progress 2003; 19(4):1109-17.

[141] KongjanP, Thong S, Kotay M, Min B, Angelidaki I. Biohydrogen Production From Wheat Straw Hydrolysate by Dark Fermentation Using Extreme Thermophilic Mixed Culture. Biotechnology and Bioengineering 2010; 105(5):899-908.

3 CONCLUDING REMARKS

In the work presented in this thesis, it was investigated the possibility of updating a rapeseed biodiesel plant, and upgrading it by integrating processing of oilseed rape residues for production of value-added products. Special merit was given to production of cellulosic ethanol, and cellulases from rape straw lignocellulose.

In a feasibility study, a scenario of expanding and upgrading an existing biodiesel plant to a state-of-the-art whole-crop oilseed rape biorefinery was presented. The results shown biorefinery platforms are nowadays taking off, and that there is presently adequate scientific and technological maturity to integrate streams for production of ethanol, biogas, superior quality animal fodder, energy, cellulolytic enzymes and chemical building blocks like methanol from biodiesel residues the whole-crop biorefinery, using both chemical, and biological pathways.

Competitive production of cellulosic ethanol from rape straw was technical optimized, by assessing the best factors and conditions for major processing steps: thermo-chemical pre-treatment by Wet oxidation, enzymatic hydrolysis with cellulases, and fermentation of C6 sugars by *Saccharomyces cerevisiae*. It was the first time that such a holistic investigation was realised for rape straw.

Pretreatment for rape straw by Wet Oxidation had very encouraging implications both for processibility of rape straw to ethanol, and exploitability of the resides from ethanol production; the optimal pre-treatment conditions provided simultaneously, the desired increased digestibility of cellulose to ethanol, and high recovery of cellulose, hemicellulose, and lignin.

Enzyme performance in enzyme saccharification was the limiting step in cellulosic ethanol production line, and the idea of creating tailor-made enzymes with better properties, using pre-treated rape straw as carbon source was developed. Best combination of carbon source from pre-treated rape straw was a mixture of C6 and C5 sugars of pre-

treated rape straw. The approach of making enzymes from rape straw had huge technological potential, since in this preliminary study enzymes of significant quality and quantity were produced by *Trichoderma reesei*. In a biorefinery perspective carbon balances showed that 20% of pre-treated rape straw needs to ber allocated for on-site enzyme production.

Using Simultaneous Saccharification and Fermentation (SSF) system is merely incompatible with *Saccharomyces cerevisiae,* since there is considerable temperature discordance between combined processes. For this, either thermophilic yeast is required to be deployed in SSF, or a different ethanol production setup. pH played a crucial role ethanol production and contamination, and careful regulation was found crucial. Finally, increment of concentration of solids in SSF was possible, but further increment needs further study.

4 FUTURE OUTLOOK

Rape straw utilization for ethanol production and other biobased products requires further both basic and applied study. It is essential to understand the structure of plant cell-walls, analyse their composition in every detail, before realizing the properties of these materials and further processing. Since rape straw is a potential substrate for ethanol production and other biofuels, low cost pre-treatment techniques like hydrothermal treatment should be studied extensively as alternatives to wet oxidation, to reduce production costs. The presence of high amounts of acetic acid in hemicellulose is an excellent opportunity to be exploited to facilitate autocatalytic chemical modifications to rape straw. Development of a new processing system equivalent to Simultaneous Saccharification and Fermentation (SSF) is required to close the gap of temperature optima of biocatalysts, as well as facilitate enzyme and year recycling. Furthermore, a techno-economic evaluation of cellulosic ethanol is required. The same is required for the bigger picture of the entire oilseed rape biorefinery. Finally, LCA analysis of the biorefinery is needed as well to evaluate the argument that there is net benefit for the environment by using rape residues for processing added-value products.

Paper I

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I WET OXIDATION PRETREATMENT OF RAPE STRAW FOR ETHANOL PRODUCTION

Contributors: Efthalia Arvaniti, Anne Belinda Bjerre, Jens Ejbye Schmidt

Abstract

Rape straw can be used for production of second generation bioethanol. In this paper we optimized the pretreatment of rape straw for this purpose using Wet oxidation (WO). The effect of reaction temperature, reaction time, and oxygen gas pressure was investigated for maximum ethanol yield via Simultaneous Saccharification and Fermentation (SSF). To reduce the water use and increase the energy efficiency in WO pretreatment features like recycling liquid (filtrate), presoaking of rape straw in water or recycled filtrate before WO, skip washing pretreated solids (filter cake) after WO, or use of whole slurry (Filter cake + filtrate) in SSF were also tested. Except ethanol yields, pretreatment methods were evaluated based on achieved glucose yields, amount of water used, recovery of cellulose, hemicellulose, and lignin.

The highest ethanol yield obtained was 67% after fermenting the whole slurry produced by WO at 205 ^{o}C for 3 min with 12 bar of oxygen gas pressure and featured with presoaking in water. At these conditions after pre-treatment, cellulose and hemicellulose was recovered quantitatively (100%) together with 86% of the lignin. WO treatments of 2-3 minutes at 205-210 ^{o}C with 12 bar of oxygen gas produced higher ethanol yields and cellulose, hemicelluloses, and lignin recoveries, than 15 minutes WO treatment at 195 ^{o}C. Also, recycling filtrate and use of higher oxygen gas pressure reduced recovery of materials. The use of filtrate could be inhibitory for the yeast, but also reduced lactic acid formation in SSF.

Keywords: wet oxidation, ethanol and rape straw, enzymatic hydrolysis

1 Introduction

Exploitation of sugars of lignocellulosic biomass resources for sustainable production of feed, energy, and fibers bespeak overcoming recalcitrance of biomass to fermenting yeasts [1]. For this purpose two strategies have been developed in the last decades. First, a pretreatment step was introduced that destroyed the coherence of biomass and improved its digestibility [2]. Secondly, hydrolytic enzymes (cellulases, hemicellulases) were introduced to hydrolyze carbohydrates of pretreated biomass to fermentable monosaccharides [3].

To support environmental sustainability of ethanol production, priority is given to use of agricultural and forest residues [4]. Rape straw is an abundant agricultural residue that has been proposed recently as feedstock for ethanol production [5]. Rape straw consists mainly of cellulose, hemicellulose, and lignin polymers, but also from other minor compounds like waxes, ashes, organic acids etc. The complete composition was been published earlier [6]. Cellulose is a highly crystalline mono-polymer of glucose, tightly packed in microfibrils, and surrounded by hemicellulose that is acetylated heteropolymers of C5 sugars (xylose, arabinose) and C6 sugars (mannose, glucose, and galactose), and lignin that is a 3D network of phenyl-propane units [7]. The enzymes needed for complete saccharification of cellulose to glucose are endoglucanases, exoglucanases and β-glucosidases that work synergistically. The enzyme digestibility of cellulose is enhanced by loosen up the structure of cellulose and removing part of the lignin and hemicellulose coatings coatings [1, 8], to allow cellulolytic enzymes to access cellulose. Such a complicate task is carried out by pretreatment.

Numerous thermo-chemical pretreatment technologies have been developed in the last decades. Examples of pretreatment methods tested on rape straw are dilute acid [9], phosphoric acid and acetone [9], hydrothermal treatment at neutral[6], acidic [10], or alkaline pH [6], wet explosion [11], ozone [6], and wet oxidation [5]. So far, optimal pretreatment method rape straw has not been assigned. Qualifications of a pretreatment method sought for ethanol production are: maximum recovery of sugars, complete and fast digestibility of pretreated rape straw by enzymes and yeasts (high product yields and productivities), and low economy (energy, water, chemicals) [8]. Lack of information in pretreatment mechanisms is underscored by use of empirical formulas like severity factors [12]. Many times the processing cost is the limiting factor in pretreatment selection, because pretreatment methods are many times energy and chemical demanding [13]. Moreover, the severity of the process destroys sugars, and forms degradation compounds inhibitory for enzymes and yeasts [14].

Wet oxidation (WO) is an aqueous high temperature high pressure pretreatment method, that uses oxidative agents. The mechanism lies on formed hydroxyl radicals, and autocatalyzing by formed

organic acids [15]. It has been tested on various feedstocks, including a preliminarily study with rape straw [5]. However, the pretreatment conditions applied on rape straw were only estimated based on optimal results on wheat straw [16]. Given that pretreatment methods are tailor-made for a given biomass [8], optimization for rape straw is required.

Due to the previous promising preliminary results of WO pretreatment on rape straw [5], we wanted to study this combination in more detail, with oxygen gas as sole chemical. The efficiency of the WO process was measured mainly by the ethanol yields, but also by the glucose yields, the amount of water used, the recovery of cellulose, and the recovery of hemicellulose and lignin. The examined pretreatment parameters were reaction temperature, oxygen pressure, reaction time, and also featured configurations like recycling filtrate, presoaking of rape straw before WO in water or filtrate, skip rinsing of pretreated rape straw after WO, and using whole slurry for ethanol production.

2 Materials and methods

2.1 Raw material

Oilseed rape straw (*Brassica Napus*, variery Carakas) was collected from fields of Hornsherred near Lyngby, Denmark in August 2007, air-dried to 90-95% humidity and stored at room temperature. Before use, the straw was reduced in 2 mm particle size by knife mill.

2.2 Pretreatment

Pretreatment experiments were carried out in a 2 L loop reactor with recirculation and stirring [17]. 60 g of dried milled rape straw were suspended in 1L of demineralized water (6% DM). The overhead chamber of 1L was either air at ambient pressure for hydrothermal treatment (control: experiment A), or impregnated with 12 bar of oxygen gas for Wet oxidation (WO; Experiment B).

Hydrothermal pretreatment (A) was the control of the wet oxidation (WO) pretreatment (B). After pretreatments A and B, fibers were separated from liquid via vacuum filtration with 0.1 mm mesh at 30 C, and filter-cake was rinsed very rapidly with 1L tap water. After separation, both filter cake and filtrate were stored in freezer (-20 ^{o}C) before further use.

In total, 15 pretreatment strategies were investigated as presented in Table 1. Experiments of same capital letter had same reaction time and temperature (experiments A to E); while subscript

characterized other parameters tested like use of oxygen at lower pressure (B_{lowP}), recycling filtrate for WO at two dilutions ($B_{recyc50}$, or $B_{recyc90}$), presoaking of fibers before WO ($B_{presoak/recyc50}$), or changing rinsing strategy of filter cake (B_{nowash}), or use of whole slurry downstream ($B_{nowash/slurry}$), as well as combinations of the above parameteres (e.g. $B_{lowP/presoak/recyc50}$).

2.3 Liquefaction and Simultaneous Saccharification and Fermentation

Simultaneous Saccharification and Fermentation (SSF) was preceded by a pre-hydrolysis (liquefaction) step [18]. For liquefaction, moist filter cake of pretreated rape straw (17% DM) was suspended in water and mixed with 15 FPU/g DM Cellubrix (Novozymes, Bagsvaerd Denmark). at 12.5% DM and at pH 4.8 in duplicates. In experiments $B_{presoak/no-wash/slurry}$ and $D_{presoak/no-wash/slurry}$ fibers were suspended in filtrate instead of water. The bottles were sealed and shaken at 50 °C and 120 rpm for 24 hours. For SSF, 20 FPU/g DM of Cellubrix L were added, together with 60 ppm urea, and 2.5 g/l dry baker's yeast (Malteserkors Gær, Denmark). The bottles were flushed with nitrogen gas, sealed with air-tight locks and shaken at 32 °C and 100 rpm. SSF with use of whole slurry (cake + filtrate) lasted for 333 hours, and SSF with use of water (no filtrate) lasted for 162 hours. Ethanol production was monitored via the CO_2 weight-loss method, and bottles were weighed every 0, 2, 5, 21, 25, and 29 hours. After the end of SSF, spent fibers were separated from beer through decanting, were dried at 105 °C for 24 hours, and milled for further analysis. The ethanol-rich beer was centrifuged at 4000 rpm for 5 minutes and stored at -18 °C for further analysis.

2.4 Analytical methods

2.4.1 Analysis of composition of raw material, filter cake and spent fibers

The composition of the raw material (app. 94% DM) was analyzed for lipophilic extraction: 5 g of dried milled raw material were boiled in Soxhlet apparatus with 1000 ml ethanol for 24 hours. After drying the fibers at 60 °C overnight and then for 1hour at 105 °C, the weight difference of the fibers gave the total lipophilic extractives. Raw material, filter-cake from pretreatment, and spent fibers after SSF were first dried overnight at 105°C and knife-milled to 0.5 mm particle size, and then analyzed via strong acid hydrolysis for total sugars and Klason lignin content [5].

Experiment label	Temperature (oC): time (min)	Oxygen gas pressure (bar)	Pretreatment and post-pretreatment handling details
No pretreatment	-	-	No pretreatment; use of dry raw fibers
A	195:10	Ambient air	Hydrothermal treatment: 6% DM in water; filtration after treatment at 30 °C, and rinsing fibers with 1L tap water (20 °C)
B	195:15	12	6% DM in water for WO, filtration after WO at 30 °C, and rinsing fibers with 1L tap water (20 °C)
B_{LowP}	195:15	8	Same as B
$B_{recyc50}$	195:15	12	6% DM in 50% diluted filtrate, otherwise same as B
$B_{recyc90}$	195:15	12	6% DM in 10% diluted filtrate, otherwise same as B
$B_{presoak/recyc50}$	195:15	12	6%DM pre-soak in 50% diluted filtrate for 20 min at 80 °C, otherwise same as B
$B_{LowP/presoak/recyc50}$	195:15	8	6%DM pre-soak in 50% diluted filtrate for 20 min at 80 °C, otherwise same as B/p
$B_{no-wash}$	195:15	12	No rinsing fibers after filtration, otherwise same as B
C	200:5	12	Same as B
D	205:3	12	Same as B
E	210:2	12	Same as B
$B_{no-wash/slurry}$	195:15	12	No rinsing fibers after filtration, after WO filtration at 30oC. Finally remixing filter cake and filtrate at 12.5%DM

$B_{presoak/no-wash/slurry}$	195:15	12	6%DM pre-soak in water for 20 min at 80 °C, after WO filtration at 30oC. Finally remixing filter cake and filtrate at 12.5%DM
$D_{presoak/no-wash/slurry}$	205:3	12	6%DM pre-soak in water for 20 min at 80 °C, after WO filtration at 30oC. Finally remixing filter cake and filtrate at 12.5%DM

Table 1 Nomenclature ***of experiments, and description of the conditions and featured configurations of the applied pretreatment***

2.4.2 Analysis of composition of pretreated filtrate and beer

The filtrate collected after pretreatment was analyzed: for total soluble sugars via weak acid hydrolysis method [19]; for free sugars (glucose, xylose, arabinose); for organic acids (formic acid, acetic acid, lactic acid, succinic acid, glycolic acid); and for furans (2-furfuraldehyde, 5-hydroxy-methylfurfuraldehyde, and 2-furoic acid) in HPLC (see below). Moreover, filtrate was analyzed on total phenolics through Prussian blue method. The beer after SSF was analyzed for free sugars, and ethanol in HPLC.

2.4.3 Enzyme assay of pretreated rape straw

The cellulase activity of Cellubrix L measured as volumetric Filter-paper activity (FPA) was 89 FPU/ml, and volumetric activity of β-glucosidase (βG) was 28 IU/ml. FPA was measured by Ghose method [20], and βG activity by Berghem method [21].

For testing enzyme digestibility of pretreated rape straw moist filter-cake was suspended at 2% DM in demineralized water, and mixed with 30 FPU/g DM Cellubrix L in a total working volume of 8 ml in triplicates. Finally, in enzyme assays of experiments $B_{no\text{-}wash/slurry}$, $B_{presoak/no\text{-}wash/slurry}$ and $D_{presoak/no\text{-}wash/slurry}$, fibers were suspended in filtrate instead of water, and filtrate corresponded to 80%v/v of the solution. The vials were sealed and shaken rotary at 50 ^{o}C for 24 hours together with enzyme blank. After the end of the assay, supernatants were measured for free sugars (glucose, xylose, and arabinose) in HPLC.

2.4.4 HPLC analysis

A Shimadzu Corp HPLC (Kyoto, Japan) system equipped with BioRad HPX-87H column (Amminex) at 63 ^{o}C, using 4 mM H_2SO_4 as eluent at 0.6 ml/min flow rate for detecting the ORGANIC ACIDSs, furans, ethanol, and sugars. The detector for furans was a Diode array SPD-M10AVP (Shimadzu Corp, Kyoto, Japan) and for the other compounds was a RID-10A RI-detector (Shimadzu Corp, Kyoto, Japan).

2.5Calculations

Cellulose recovery (glucose) and **hemicellulose recovery** (xylose, arabinose) after pre-treatment were calculated as a percentage of the raw material sugars added in the pretreatment reactor. The recoveries were calculated for both soluble and insoluble fractions (cake, filtrate):

$$Recovery\ \left(\frac{g\ sugar\ in\ pretreated\ material}{\text{g sugar in untreated material}}\%\right) = \frac{C_{sugar} \times \mathrm{R_s}}{H_f \times C_{C/H}} \times 100\% \qquad (1)$$

Where C_{sugar} $(\frac{g\ sugar}{g\ pretreated\ solids})$, is the concentration of glucose, xylose and arabinose; R_s $(\frac{g\ Pretreated\ solids}{g\ \ untreated\ material})$ is the recovery of insoluble solids after pretreatment; $C_{C/H}$ $(\frac{g\ Carbohydrate}{g\ Raw\ material})$ is the concentration of carbohydrates (e.g. cellulose, hemicellulose) in the raw material; and H_f is the Hydration factor of each sugar$(\frac{g\ sugar}{g\ Carbohydrate})$; for glucose H_f is 1.100, and for xylose and arabinose H_f is 1.136.

Glucose yield % in enzyme assays was calculated in a percentage basis of the glucose potential of added pretreated biomass (100%):

$$Y_{Gl}(\frac{g\ \text{released}\ glucose\ after\ enzyme\ hydrolysis}{\text{g glucose potential of added in enzyme hydrolysis}}) = \frac{C_{Gl}}{C_{Cel} \cdot H_f} \cdot 100\% \qquad (2)$$

C_{Gl} is the released glucose in enzyme hydrolysis experiments $(\frac{g}{l})$, C_{Cel} is the concentration of cellulose added in the enzyme assays $(\frac{g}{l})$, and H_f is the hydration factor of glucose $(\frac{g\ glucose}{g\ cellulose})$ equal to 1,1.

Under the same principle, **cellobiose yield** % and **xylose yield %** was calculated, as the percentage of the released cellobiose and xylose after enzyme hydrolysis of the cellobiose and xylose potential of cellulose and hemicellulose respectively (100%). In these two cases, H_f was 1,05, and 1,136 respectively.

Ethanol yield after SSF was calculated in a percentage basis on the percentage of the theoretical ethanol potential of cellulose of raw material:

$$Y_{EtOH}(\frac{g\ ethanol\ produced}{\text{g theoritical ethanol potential of added cellulose in SSF}}\%) = \frac{\frac{C_{EtOH} \cdot Y_s}{C_s}}{C_{Cel}^{RM} \cdot H_f \cdot Y_{EtOH}^0} \cdot 100\% \qquad (3)$$

Where C_{EtOH} measured $(\frac{g}{l})$, Y_s is the yield of solid biomass from pretreatment step $(\frac{g\ Total\ Solids}{g\ Raw\ material})$, C_s is the DM of biomass in SSF $\left(\frac{g\ Total\ Solids}{l}\right)$, C_{Cel} is the cellulose content of raw material $(\frac{g\ cellulose}{g\ RM})$, H_f is the hydration factor of glucose $(\frac{g\ glucose}{g\ cellulose})$ which is 1.1, and Y_{EtOH}^0, is the theoretical ethanol yield of glucose $(\frac{g\ ethanol}{g\ glucose})$ 0.51.

Progress of ethanol fermentation is calculated from equivalents of CO_2 vent during fermentation or SSF. Ethanol equivalent for each gram of CO_2 vented is 1,045. From this amount of ethanol, ethanol yields are calculated as described above.

Intensity of pretreatment method was assessed by the empirical **Severity factor** R_0 (eq 4), that translates pretreatment temperature and reaction time into impact factors on deconstruction and alteration of lignocellulosic biomass [22]:

$$R_o = exp\frac{T-100}{14.75} \times t \quad (4)$$

Where T is reaction temperature and t is reaction time.

Standard deviation was used for analyzing the dispersion of replicate experiments, and stems all graphic illustrations in figures. The standard deviation was calculated using STDEVA formula of Excel software.

One-way analysis of variance (ANOVA) analyzed data for 5% and 10% significance level by grouping pretreatment parameters (treatments) at different conditions (levels). In the case that group contained more than two levels, and results were significantly different, **Newman and Keuls post-hoc statistical analysis** tool was applied for 5% or 10% significance level, to identify in which levels of that treatment were significantly different. Newman and Keuls was calculated with DSAASTAT macro v. 1.101 (Perugia, Italy) in Excel software.

Correlation coefficient (COCO) was applied between two arrays of variables. Here is used to relate either a dependent to an independent variable, or two dependent variables, using a model from Excel software Microsoft office 2007.

3 Results and discussion

Following experiments are categorized on oxygen gas pressure, recycling of filtrate, presoaking of rape straw before WO, combinational change of temperature and reaction time, skip of rinsing, use of filtrate for ethanol production (see Table 1).

3.1 The effect of oxygen

To evaluate the effect of oxygen gas pressure on the pretreatment and the digestibility of fibers the following experimental setup was used. Low and high oxygen pressure of gas in WO were compared in experimental pairs of B_{lowP} (8 bars) and B (12 bars, see Table 1), and $B_{lowP/presoak/recyc50}$ (8 bars) and $B_{presoak/recyc50}$ (12 bars). Moreover, experiment B that is the base case of WO was compared to experiment A that is hydrothermal treatment, and to Control experiment that received no treatment.

The results of the recoveries of cellulose and lignin were significantly different for B_{lowP} and B, but not for hemicellulose. Cellulose recovery increased from 96% to 99% by decreasing pressure from 12 bar to 8 br (in B to B_{lowP} respectively, see Figure 1A), lignin recovery increased from 58% to 68% (Figure 1C), whereas hemicellulose recovery ranged 71%-77% (Figure 1B). For the pair $B_{presoak/recyc50}$ and $B_{lowP/presoak/recyc50}$ that combined recycling of the filtrate and presoaking of rape straw, cellulose and lignin recovery again significantly increased from 84% to 101 for cellulose by lowering oxygen pressure from 12 bar to 8 bar (in $B_{presoak/recyc50}$ to $B_{lowP/presoak/recyc50}$respectively), and from 77% to 92% for lignin respectively. Hydrothermal treatment (exp. A) had comparable recoveries with WO (exp. B) with cellulose at 96% and hemicellulose at 89%. However, the recovery of lignin was much higher (95%) than B (see Figure 1C).

Digestibility of cellulose of pretreated rape straw is shown in Figure2. The yields for increasing oxygen pressure significantly increased from 45% to 51% from B to B_{lowP}, and decreased from 65% to 56% for $B_{presoak/recyc50}$ and $B_{lowP/presoak/recyc50}$ respectively. Glucose yield of hydrothermal treatment was as low as 48%, and without rape straw cellulose without pretreatment was hydrolyzed only by 16%. In the enzymes assays except glucose (Figure 2), also cellobiose was detected, which is a dimmer of glucose and main substrate of βG. Cellobiose accounted for 17-30% of yield and 1.6-4.0 g/l in enzyme assays in B, B_{lowP}, $B_{presoak/recyc50}$, and $B_{lowP/presoak/recyc50}$. However, in the same ranges was observed for the total of experiments. Cellobiose accumulation is more likely caused by inhibition of βG. Back in 2004 Varga et al. (2004) had reported that glucose yield of WO pretreated corn stover in the same assay increased only by 2% when Cellubrix was supplemented by βG [23]. Potential inhibition of Cellubrix at high sugar concentration applications needs to be studied further.

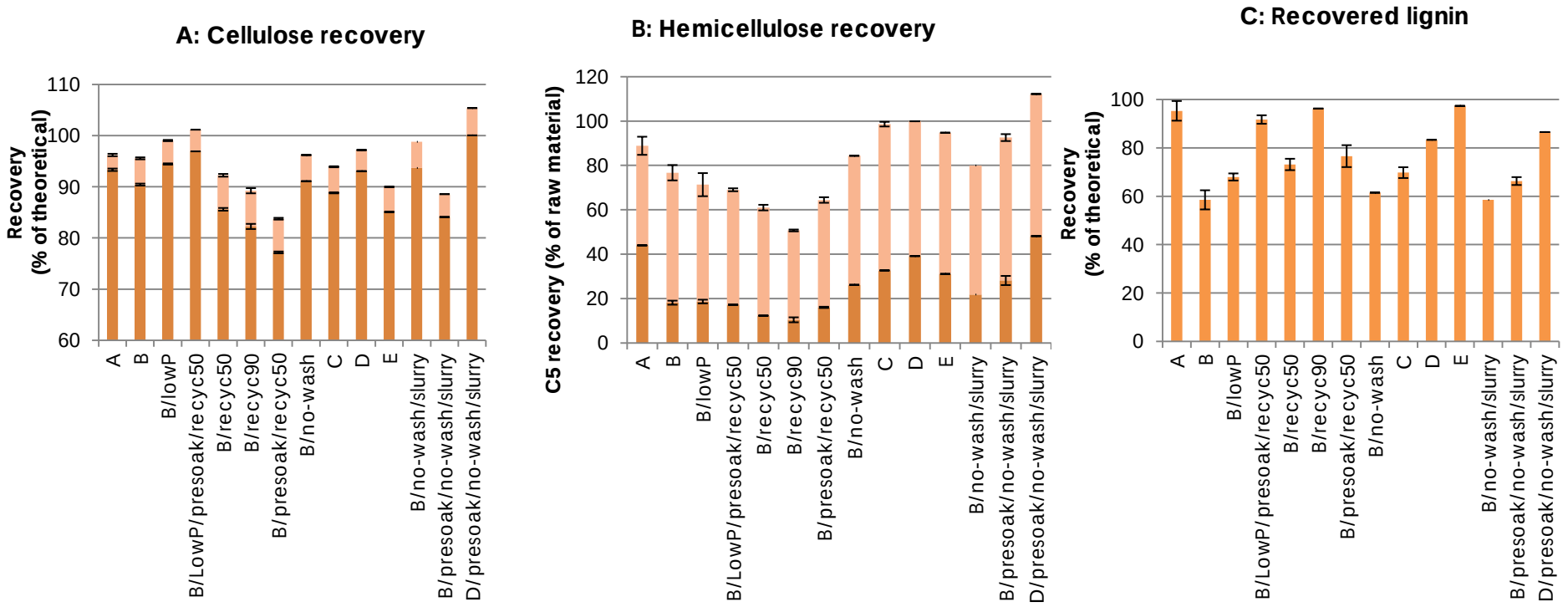

Figure 1: **Recovered main components of rape straw after pretreatment: A) recovered cellulose both soluble (dark) and insoluble fractions (light); B) recovered C5 sugars, both insoluble (dark) and soluble (light) fractions; and C) recovered insoluble lignin. The error bars show the standard deviation of the duplicates.**

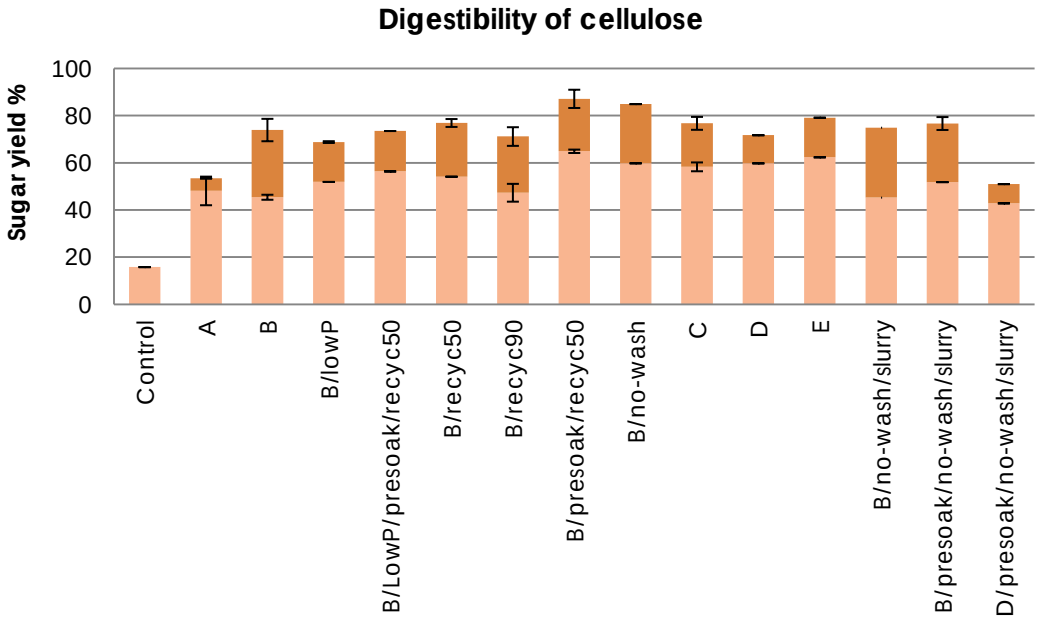

Figure 2. Glucose yields (light) and cellobiose yields (dark) after enzymatic saccharification of pretreated rape straw by Cellubrix L after 24 hours. In $B_{no\text{-}wash/slurry}$, $B_{presoak/no\text{-}wash/slurry}$, and $D_{presoak/no\text{-}wash/slurry}$ experiments whole slurry was used, otherwise moist filtered pretreated rape straw was suspended in buffer. The error bars show the standard deviation of the duplicates

In SSF experiments where glucose is *insitu* consumed by yeast, cellobiose was not detected. Thereby βG was no evidence for inhibition [24], and it was decided to use Cellubrix for all SSF experiments. Ethanol yields were not significantly different for B_{lowP} and B ranging 61-67%, or for $B_{presoak/recyc50}$ and $B_{lowP/presoak/recyc50}$ ranging 57-58% (see Figure 3). The ethanol yield for hydrothermal treatment was 51%, and for the control was 18%. Previous reports about optimal hydrothermal treatment of rape straw for ethanol production are contradictory[25, 26]. For this conditions of hydrothermal treatment were selected based on optimal of wheat straw [16]. The statistical analysis of B_{lowP} and B, as well as of $B_{presoak/recyc50}$B/rs and $B_{lowP/presoak/recyc50}$ from ANOVA and Newman Keuls post-hoc analysis are listed in Table 2 and Table 3.

3.2 Recycling

Goal of this experiment is to reduce the amount of water used in WO pretreatment by recycling filtrate. For this, experiments B (no recycle), $B_{recyc50}$ (50% recycle of filtrate), and $B_{recyc90}$ (90% recycle of filtrate) are compared.

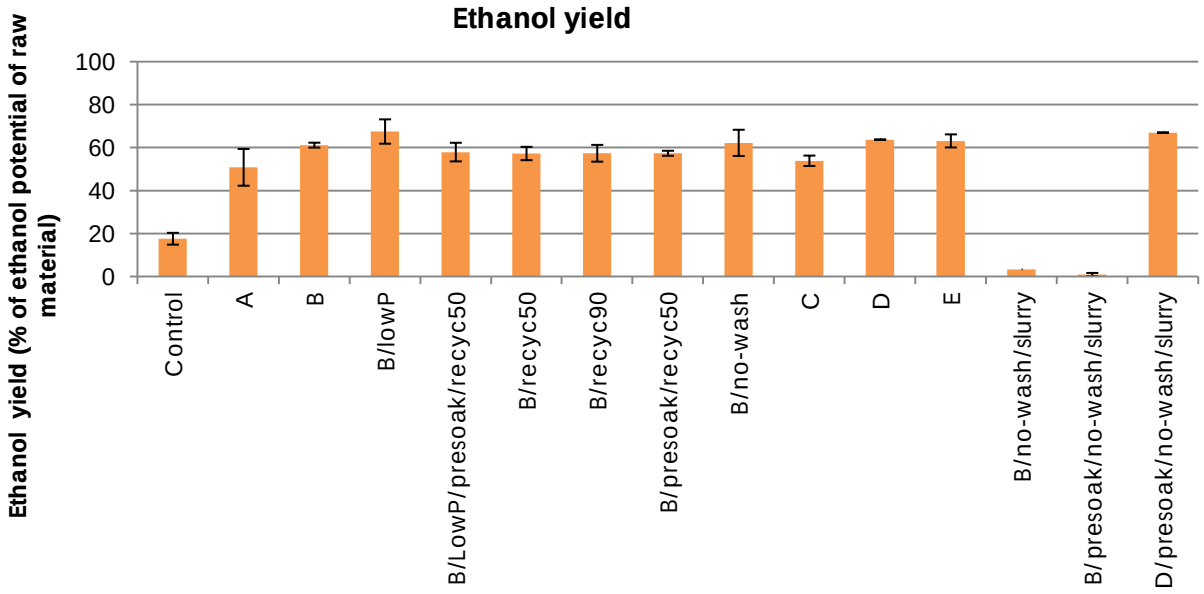

Figure 3. Ethanol yield after Simultaneous Saccharification and Fermentation (SSF) of pretreated rape straw C6 sugars by Cellubrix L and baker's yeast. In experiments $B_{no\text{-}wash/slurry}$, $B_{presoak/no\text{-}wash/slurry}$, and $D_{presoak/no\text{-}wash/slurry}$ whole slurry was used and SSF lasted for 333 hours, whereas in other experiments filtered pretreated rape straw was suspended in water and SSF lasted for 162 hours.

Cellulose recovery was not significantly different for B, $B_{recyc50}$, and $B_{recyc90}$, varying at 89-96% respectively (see Table 2 and Figure 1A). Recovery of hemicellulose (see Figure 1B) significantly decreased by recycling from 77% (no recycling) to 51% (90% recycle). Lignin recovery was significantly different also and increased by increasing recycling fraction from 58% (no recycling) to 73%, and 96% (90% recycling, see Table 3 and Figure 1C).

The most common soluble degradation products found in the filtrates after pretreatment are presented in Figure 4. The pH of filtrate after B, $B_{recyc50}$, and $B_{recyc90}$ pretreatment ranged 3.4-3.5 (data not shown). There was significant difference in concentrations of formic acid and acetic acid for the B, $B_{recyc50}$, $B_{recyc90}$ experiments (see Table 3, and Figure 4A), where both acids increased by increasing recycling fraction of filtrate (See Figure 5A). Other acids like glycolic acid and succinic acid were in the order of 0.3 g/l and 0.2 g/l (data not shown). Formic acid and acetic acid are expected to come primarily from hydrolysis of hemicellulose and uronic acids (pectin) [6] under low pH [8]. For raw rape straw Alexander et al (1987) accounted acetic acid and formic acid for 5% [6]. For furfural and soluble phenolics (see Figure 4B) there was significant difference for B, $B_{recyc50}$, and $B_{recyc90}$ (see Table 2 and Table 3). Furfural increased from 0.3 g/l to 1.1 g/l by recycling, and soluble phenolics increased from 1.8 g/l to 2.4 g/l.

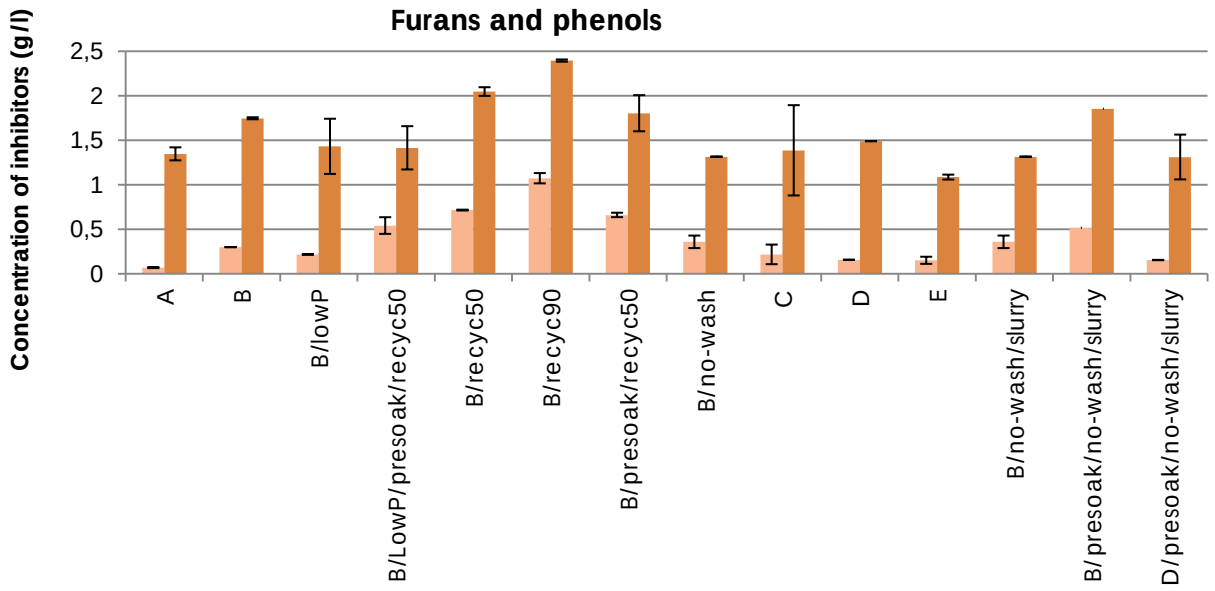

Figure 4. Concentration of A) formic acid (grey) and acetic acid (black), and B) furfural (grey) and phenolic compounds (black) found in filtrate after pretreatment experiments. Filtrates from B were recycled to experiments $B_{recyc50}$, $B_{recyc90}$, $B_{presoak/recyc50}$ and $B_{LowP/presoak/recyc50}$. Filtrates from $B_{no-wash/slurry}$, $B_{presoak/no-wash/slurry}$, and $D_{presoak/no-wash/slurry}$ were used together with filter cakes in enzyme assays and SSF experiments. Error bars show the standard deviation of duplicates.

Correlation coefficient (COCO) of soluble organic acids found in filtrate with insoluble lignin in B, $B_{recyc50}$ and $B_{recyc90}$ pretreatments was close to 0.93, COCO of organic acids with soluble C5 sugars was -0.96, and also COCO of soluble phenolic compounds with insoluble lignin was close to 0.96. Moreover, when the amounts of soluble organic acids and soluble phenols of B filtrate were subtracted from the resulting recycled filtrate $B_{recyc50}$ or $B_{recyc90}$, the correlations were unchanged. Finally COCO of formic acid with acetic acid was 0.98. COCOs indicate that organic acids have strong affiliation with insoluble hemicellulose and lignin, and that organic acids increased with high recovery of lignin. The latter observation of concurrent increase of soluble product and insoluble parent compound was observed also for (soluble) phenolics/(insoluble) lignin that had a COCO of 0.96.

Table 2 Results from ANOVA analysis. Compared groups (independent variables) were Pressure, Recycling, Presoaking, Filtrate content in the insoluble solids, and combinations of temperature and time. Dependent variables are the total recovery (soluble and insoluble) of pretreated rape straw cellulose, hemicellulose, the recovery of insoluble hemicellulose and lignin, the glucose yield, the ethanol yield, and concentration of degradation products found in the filtrate of pretreated rape straw. **=significantly different values from ANOVA by 5% significance, *= significant different values , 10% significance, ns= Not significant difference, NA=Not analyzed

Analyzed parameters	Oxygen gas pressure		Recycling filtrate	Presoaking rape straw	Temperature/Time combinations
Pretreatment groups	$B + B_{LowP}$	$B_{presoak/recyc50} + B_{LowP/presoak/recyc50}$	$B + B_{recyc50} + B_{recyc90}$	$B_{recyc50} + B_{presoak/recyc50}$	B + C + D + E
Cellulose recovery %	*	**	ns	*	ns
Hemi-cellulose recovery %	ns	ns	**	ns	**
Insoluble Lignin recovery %	*	*	**	ns	**
Glucose yield %	**	**	*	**	**
Ethanol yield %	ns	ns	ns	ns	**
VFA concentration (g/l)	ns	ns	**	ns	ns
Furfural concentration (g/l)	**	ns	**	*	ns
Phenolics (g/l)	ns	ns	**	ns	ns

Table 3 Summarizing table containing significantly different data (ANOVA) after being post-hoc analyzed by Newman-Keuls statistical tool, as well as raw data (italic font), all presented in descending order of magnitude a to d, with a the highest and d the lowest value. ns= not significant according to ANOVA (table 2).

Analyzed parameters	Oxygen gas pressure				Recycling filtrate			Presoaking rape straw				Filtrate in pretreated solids			Temperature/Time combinations					
Analyzed grouped treatments	B	B_{LowP}	$B_{presoak/recyc50}$	$B_{LowP/presoak/recyc50}$	B	$B_{recyc50}$	$B_{recyc90}$	$B_{recyc50}$	$B_{presoak/recyc50}$	$B_{no-wash/slurry}$	$B_{presoak/no-wash/slurry}$	B	$B_{no-wash}$	$B_{no-wash/slurry}$	B	C	D	E	$B_{presoak/no-wash/slurry}$	$B_{presoak/no-wash/slurry}$
Cellulose recovery %	b	a	b	a	ns			a	b	a	b				ns				b	a
Hemicellulose recovery %	ns		ns		a	b	c	ns		b	a				b	a	a	a	b	a
Insoluble Lignin recovery %	b	a	b	a	c	b	a	ns		b	a				d	c	b	a	b	a
Glucose yield %	a	b	a	b	ns			ns		b	a	b	a	b	ns				a	b
Ethanol yield %	ns		ns		ns			ns		a	b	a	a	b	a	b	a	a	b	a
VFA concentration (g/l)	ns		ns		c	b	a	ns		b	a				ns				a	b
Furfural concentration (g/l)	a	b	ns		c	b	a	a	b	a	b				ns				a	b

Phenolics (g/l)	ns	ns	c	b	a	ns	b	a				ns	a	b

Acetic acid and formic acid are not only hydrolysis products, but also degradation products of furans and phenolics that are in turn degradation products of carbohydrates and lignin [16]. The fractionation pattern of lignin during chemical pre-treatment is: Solid lignin → Soluble lignin → Phenolic derivatives → Carboxylic acids → CO_2 + H_2O [16]. We observed an increase of intermediate degradation products of lignin concurrently with the recovery lignin at low pH (recycling the filtrate) that has been reported previously [27], and could be explained by the production of "pseudolignin" by condensed phenolics and sugars.

Glucose yields when filtrate was recycled in WO were found significantly highest (see Table 2) only for $B_{recyc50}$ 54% (Figure 2). The ethanol yields were found not significantly different ranging 57-61% (Figure 3). Previous studies have shown that delignification improves glucose saccharification [8, 28]. In hereby study such correlation was not observed. The glucose yields increased significantly by 50% filtrate recycling, while the lignin increased significantly from 59% (no recycling) to 73% (50% recycling).

3.3 Presoaking of rape straw before WO

The goal of this experiment is to improve the efficiency of energy use by presoaking rape straw before WO in water (neutral pH) or in filtrate (pH 3.5). For this (see Table 1), a) $B_{no\text{-}wash/slurry}$ (no presoaking) is compared to $B_{presoak/no\text{-}wash/slurry}$ (presoaking in water); b) $B_{recyc50}$ (no presoaking but WO in recycled filtrate) is compared to $B_{presoak/recyc50}$ (presoaking and WO in recycled filtrate).

Recovery of cellulose significantly decreased with use of presoaking in recycled filtrate ($B_{presoak/recyc50}$) compared to no-presoaking WO ($B_{recyc50}$) from 92% to 84% (see Figure 1A), whereas hemicellulose, and lignin, were not significantly altered by presoaking. Also furfural was significantly higher by presoaking (0.71 g/l, see Figure 4). But these results were not in line with the known high reactivity of hemicellulose (the parent compound) and furfural (its degradation product) under low pH [29]. Also in previous results presoaking at acidic conditions hydrolyzed more hemicelluloses than control [30]. Finally, glucose yields were significantly higher with presoaking 65% (presoaking in filtrate). Finally, the ethanol yields were 57% for both cases.

$B_{no\text{-}wash/slurry}$ and $B_{presoak/no\text{-}wash/slurry}$ was not replicated; thereby statistical analysis was limited. By featuring presoaking in water the recovery of cellulose decreased from 98% to 89%, recovery of hemicellulose increased considerably from 82% to 92%, and of lignin increased slightly from 60% to 66% respectively. Glucose yield increased by presoaking in water from 45% to 52%, and ethanol

yield was 3% and 1% respectively. The low ethanol yields are presumably the result of the inhibitory effect of filtrate in yeast during SSF.

Presoaking either in water or filtrate reduced recovery of cellulose, and increased glucose yields. However presoaking is not attractive if cellulose is degraded. Low recovery of cellulose is accounted for low recovery of glucose. This glucose however might be part of hemicellulose structure instead [31]. Alexander et al. has pointed out that hemicellulose origin of glucose might account for as high as 10% of total glucose of rape straw [6].

3.4 Filtrate concentration in pretreated rape straw

The goal is to improve efficiency of water used after pretreatment by blending pretreated cake with filtrate. For this control experiment B (rinsed cake) is compared to non-rinsed pretreated cake ($B_{no\text{-}wash}$), and $B_{no\text{-}wash/slurry}$ where the whole slurry (cake + filtrate) is used.

Experiments $B_{no\text{-}wash}$ and $B_{no\text{-}wash/slurry}$ are not replicated, and therefore statistical analysis is limited. The degradation products in filtrate were similar since pretreatment conditions were kept the same. However, the dilution factor of filtrate in the assays was different. Glucose yields of $B_{no\text{-}wash}$ that skip washing was higher (60%, see Figure 2) than that of control (B) and $B_{no\text{-}wash/slurry}$ (45% both. The enzyme assay of $B_{no\text{-}wash/slurry}$ that used filtrate contained at time zero 0.9 g/l cello-oligomers, 0.2 g/l glucose, 4.6 g/l xylooligomers, 1.2 g/l xylose, 1.0 g/l arabinooligomers, 0.2 g/l arabinose, 1.6 g/l formic acid, 1.9 g/l acetic acid, 0.4 g/l furfural, and 1.3 g/l soluble phenolics (data not shown). All these components are known inhibitors for cellulolytic enzymes [30, 32-35], and is believed that inhibited enzymes of $B_{no\text{-}wash/slurry}$ enzymes and caused reduction of the glucose yield.

Skip of rinsing did not affect ethanol yields. However, use of filtrate in SSF ($B_{no\text{-}wash/slurry}$) considerably reduced ethanol yield (3%) compared to control B and unrinsed cake ($B_{no\text{-}wash}$) that ranged 61-62%. The low ethanol yield is attributed to enzyme and yeast inhibition from filtrate. After SSF, the pH was around 3.9 and the concentration of formic acid was 1.5 g/l, and of acetic acid 3.3 g/l. It has been reported that *S. cerevisiae* can ferment glucose at pH below 3, but presence of ORGANIC ACIDs radically decreases yeast tolerance at low pH [36]. Moreover, filtrate contained 0.4 g/l furfural before SSF, and Delgenes et al. (1996) [37] reported that furfural at 0.5 g/l reduced 53% of growth of *S. cerevisiae* and 57% of ethanol production.

Finally, after SSF lactic acid was detected at 6.3, 0.2, and 0 g/l for B, Bw/1 and $B_{no\text{-}wash/slurry}$ experiments. It appears that components present in SSF of unrinsed cake ($B_{no\text{-}wash}$) or whole slurry ($B_{no\text{-}wash/slurry}$) inhibited production of lactic acid, and presumably lactic acid bacteria (LAB). Presence of LAB was not proved, but they are common contamination in dry baker's yeast

formulations [38]. Lactic acid production was detected in all SSF flasks that used rinsed pretreated rape straw (like for example B), in amounts around 6-8 g/l (data not shown) that was estimated to account for 10% of added total glucose. Lactic acid however, was not detected in enzyme assays indicating that LAB were inoculated together with the yeast in SSF.

Summarizing, the use of non rinsed pretreated rape straw in SSF represented the best case for ethanol production, since allowed ethanol fermentation, but restricted spreading contamination by LAB. With this setup about 55 liters of water were saved per liter of ethanol (data not shown).

3.5 Reaction time and temperature

The goal is to estimate the best WO reaction temperature and reaction time for rape straw among B (195 ^{o}C, 15 minutes), C (200 ^{o}C, 5 minutes), D (205 ^{o}C, 3 minutes), and E (210 ^{o}C, 2 minutes) conditions, with B as reference. The severity factors (CS) for B, C, D, and E experiments were 3.1, 0.6, 0.7, and 0.4 respectively (data not shown). Also, experiments $B_{presoak/no\text{-}wash/slurry}$ and $D_{presoak/no\text{-}wash/slurry}$ that are featured with presoaking and use the whole slurry (cake + filtrate) after pretreatment are compared. Experiments $B_{presoak/no\text{-}wash/slurry}$, D, and $D_{presoak/no\text{-}wash/slurry}$ are not replicated in pretreatment; therefore their statistical analysis is limited.

Cellulose recovery was not significantly different (see Table 2) for B, C, D, and E experiments, ranging from 90-97% (see Figure 1A). Recovery of C5 sugars was significantly different only for B (see Table 3) where total hemicellulose recovery was 77% (see Figure 1B), while for C, D, and E ranged 95-99%. Regarding recovery of insoluble hemicellulose significant maximum was for D (39%). The correlation coefficient of the insoluble hemicellulose with CS was -0.94, in line with the predictions of the models of Overend (1987) on solubilization of xylan during hydrothermal treatments [22]. Also recovery of lignin was significantly different in all the experiments; at the lowest was 59% in experiment B and increased to 70%, 83%, and 97% for C, D, and E respectively (see Figure 1C).

Comparing the results of WO with other studies for rape straw [10, 25], all examined WO conditions (B, C, D, and E) exhibited higher glucose yields, including those for alkaline WO [5]. The presented results were only comparable to application of a two-step wet explosion pretreatment method [39]. Glucose yields for B conditions were significantly lower (45%) than C, D, and E conditions ranging from 58-62%. For experiment featuring presoaking the glucose yield of $B_{presoak/no\text{-}wash/slurry}$ was 52%, compared to $D_{presoak/no\text{-}wash/slurry}$ that was only 43%. In this study, despite the difference among the CS of B, C, D, and E experiments, the glucose yields were not significantly different. Thereby the suggested direct correlation of high CS with high projected glucose yields [40] was not verified. Moreover, as mentioned in paragraph 3.2, despite previous studies, removal of lignin was not also important for achieving high glucose yields. The significantly highest glucose yields were 60% and

62% and were observed for D and E WO conditions, for which the highest lignin recovery was observed also 83% and 97% respectively.

The ethanol yields for C (54%) experiment were significantly lower than B, D, and E that ranged 61-64% (see Figure 3). For experiments featuring use of whole slurry, the ethanol yields of $B_{presoak/no-wash/slurry}$ were 1%, compared to 67% for $D_{presoak/no-wash/slurry}$. The progress of SSF after adding the yeast with whole slurry $B_{presoak/no-wash/slurry}$, and $D_{presoak/no-wash/slurry}$, and with non-presoaked rinsed cake suspended in water B, and D is illustrated in Figure 5. The ethanol productivity of presoaked whole slurry $D_{presoak/no-wash/slurry}$ was highest after 164 hours, and that of non-presoaked cake D after 26 hours. After short WO conditions ($D_{presoak/no-wash/slurry}$) on rape straw, fermentation (in SSF) of whole slurry achieved a final ethanol yield of 67% after 333 hours. This ethanol yield is comparable to control (D) that didn't use filtrate but was achieved in 168 hours. The difference in ethanol yields are presumably caused by chemical inhibitors present in the filtrate. The filtrate of $B_{presoak/no-wash/slurry}$ contained among others 1.7 g/l formic acid, and 2.2 g/l acetic acid, 0.5 g/l furfural and 1.8 g/l soluble phenolics, while the filtrate of $D_{presoak/no-wash/slurry}$ contained 43% less organic acids, 70% less furfural, and 29% less phenolics (see Figure 4).

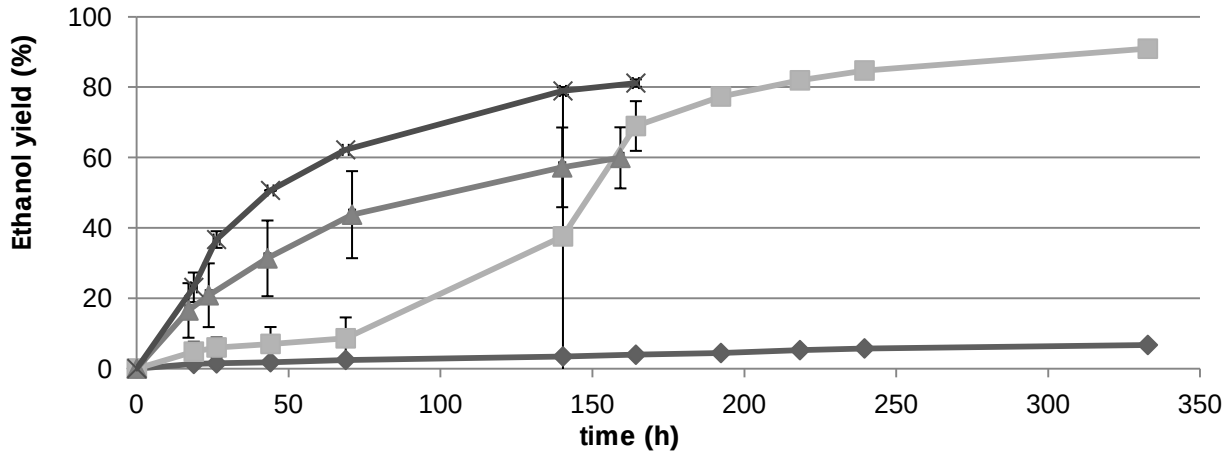

Figure 5 Ethanol yield progress in SSF experiments run of pretreated rape straw fibers suspended in water B (triangle), and D (X symbol), and pretreated rape straw fibers suspended in filtrate $B_{presoak/no-wash/slurry}$ (rhombe) and $D_{presoak/no-wash/slurry}$ (square). The error bar shows the standard deviation of the duplicates.

4 Conclusions

- WO pretreatment methods of 2-3 minutes at 205-210 °C with 12 bar of oxygen gas pressure resulted in higher glucose and ethanol yields, and in higher recovery of cellulose, hemicellulose, and lignin compared to 15 minutes treatment,. High recovery of sugars and lignin resulted in marginal production of degradation products in the filtrate.
- The highest ethanol yield achieved was 67% by combining a two step pretreatment process, where a soaking step at 80 °C for 20 min precedes WO at 205 °C, with 12 bar oxygen for 3 minutes. Under these pre-treatment conditions, cellulose and hemicellulose was recovered quantitatively together with 86% of the lignin. Therefore, for achieving high glucose yield it was not found necessary to remove large part of lignin.
- In enzyme assays β-Glucosidase of Cellubrix was inhibited by glucose evident by accumulation of cellobiose. However, such a phenomenon was not observed in SSF where glucose is readily consumed by yeast.
- Skip rinsing technique and use of whole slurry for ethanol production reduced production of lactic acid, and in this setup would save 55 liter and 80 liter of water per liter of ethanol .
- Recycling of filtrate in WO increased lignin recovery and reduced hemicellulose recovery. As a result recycling increased degradation products, and glucose yields were lower for 90% recycling glucose yields than 50% recycling. The ethanol yield however was not influenced.
- Use of 12 bar oxygen gas pressure instead of 8 bar improved ethanol yields, but decreased cellulose recovery.

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References

[1] Himmel ME, Ding S, Johnson DK, Adney WS, Nimlos MR, Brady JW, et al. Biomass recalcitrance: Engineering plants and enzymes for biofuels production. Science 2007; 315: 804-7.

[2] Chandra RP, Bura R, Mabee WE, Berlin A, Pan X, Saddler JN. Substrate pretreatment: The key to effective enzymatic hydrolysis of lignocellulosics?. Biofuels 2007; 108: 67-93.

[3] Rosgaard L, Pedersen S, Langston J, Akerhielm D, Cherry JR, Meyer AS. Evaluation of minimal Trichoderma reesei cellulase mixtures on differently pretreated barley straw substrates. Biotechnol Prog 2007; 23: 1270-6.

[4] Scarlat N, Martinov M, Dallemand JF. Assessment of the availability of agricultural crop residues in the European Union: Potential and limitations for bioenergy use. Waste Manage 2010; 30: 1889-97.

[5] Petersson A, Thomsen MH, Hauggaard-Nielsen H, Thomsen AB. Potential bioetanol and biogas production using lignocellulosic biomass from winter rye, oilseed rape and faba bean. Biomass & Bioenergy 2007; 31: 812-9.

[6] Alexander BW, Gordon AH, Lomax JA, Chesson A. Composition and Rumen Degradability of Straw from 3 Varieties of Oilseed Rape Before and After Alkali, Hydrothermal and Oxidative Treatment. J Sci Food Agric 1987; 41: 1-15.

[7] Olsson L, Jørgensen H, Krogh KBR, Roca C. Polysaccharides: structural diversity and functional versatility. In: Dumitriu S, editor. , New York: Marcel Dekker; 2005, p. 957-993.

[8] Yang B, Wyman CE. Pretreatment: the key to unlocking low-cost cellulosic ethanol. Biofuels Bioproducts & Biorefining-Biofpr 2008; 2: 26-40.

[9] Jeong TS, Um BH, Kim JS, Oh KK. Optimizing Dilute-Acid Pretreatment of Rapeseed Straw for Extraction of Hemicellulose. Appl Biochem Biotechnol 2010; 161: 22-33.

[10] Lu XB, Zhang YM, Angelidaki I. Optimization of H2SO4-catalyzed hydrothermal pretreatment of rapeseed straw for bioconversion to ethanol: Focusing on pretreatment at high solids content. Bioresour Technol 2009; 100: 3048-53.

[11] Luo G, Talebnia F, Karakashev D, Xie L, Zhou Q, Angelidaki I. Enhanced bioenergy recovery from rapeseed plant in a biorefinery concept. Bioresour Technol 2011; 102: 1433-9.

[12] Abatzoglou N, Chornet E, Belkacemi K, Overend RP. Phenomenological Kinetics of Complex-Systems - the Development of A Generalized Severity Parameter and Its Application to Lignocellulosics Fractionation. Chemical Engineering Science 1992; 47: 1109-22.

[13] Kazi FK, Fortman JA, Anex RP, Hsu DD, Aden A, Dutta A, et al. Techno-economic comparison of process technologies for biochemical ethanol production from corn stover. Fuel 2010; 89: S20-8.

[14] Klinke HB, Olsson L, Thomsen AB, Ahring BK. Potential inhibitors from wet oxidation of wheat straw and their effect on ethanol production of Saccharomyces cerevisiae: Wet oxidation and fermentation by yeast. Biotechnol Bioeng 2003; 81: 738-47.

[15] Mcginnis GD, Wilson WW, Mullen CE. Biomass Pretreatment with Water and High-Pressure Oxygen - the Wet-Oxidation Process. Industrial & Engineering Chemistry Product Research and Development 1983; 22: 352-7.

[16] Klinke HB, Ahring BK, Schmidt AS, Thomsen AB. Characterization of degradation products from alkaline wet oxidation of wheat straw. Bioresour Technol 2002; 82: 15-26.

[17] Bjerre AB, Olesen AB, Fernqvist T, Ploger A, Schmidt AS. Pretreatment of wheat straw using combined wet oxidation and alkaline hydrolysis resulting in convertible cellulose and hemicellulose. Biotechnol Bioeng 1996; 49: 568-77.

[18] Varga E, Klinke HB, Reczey K, Thomsen AB. High solid simultaneous saccharification and fermentation of wet oxidized corn stover to ethanol. Biotechnol Bioeng 2004; 88: 567-74.

[19] Bjerre AB, Ploeger A, Simonsen T, Woidemann A, Schmidt AS. Quantification of solubilised hemicellulose from pretreated lignocellulose by acid hydrolysis and high performance liqui chromatography 1996; Risoe-R-855.

[20] Mandels M, Andreotti R, Roche C. Measurement of Saccharifying Cellulase. Biotechnol Bioeng 1976: 21-33.

[21] Berghem LER, Pettersson LG, Axiofredriksson UB. Mechanism of Enzymatic Cellulose Degradation - Purification and Some Properties of 2 Different 1,4-Beta-Glucan Glucanohydrolases from Trichoderma-Viride. European Journal of Biochemistry 1976; 61: 621-30.

[22] Overend RP, Chornet E. Fractionation of Lignocellulosics by Steam-Aqueous Pretreatments. Philosophical Transactions of the Royal Society of London Series A-Mathematical Physical and Engineering Sciences 1987; 321: 523-36.

[23] Varga E, Schmidt AS, Reczey K, Thomsen AB. Pretreatment of corn stover using wet oxidation to enhance enzymatic digestibility. Appl Biochem Biotechnol 2003; 104: 37-50.

[24] Philippidis GP, Smith TK. Limiting Factors in the Simultaneous Saccharification and Fermentation Process for Conversion of Cellulosic Biomass to Fuel Ethanol. Appl Biochem Biotechnol 1995; 51-2: 117-24.

[25] Castro E, Díaz MJ, Cara C, Ruiz E, Romero I, Moya M (2011). Dilute acid pretreatment of rapeseed straw for fermentable sugar generation. Bioresource Technology 102, 1270-1276

[26] Targonsky Z. Autohydrolysis Extraction Process As A Pretreatment of Lignocelluloses for Their Enzymatic-Hydrolysis. Acta Biotechnol 1985; 5: 353-61.

[27] Martin C, Klinke HB, Thomsen AB. Wet oxidation as a pretreatment method for enhancing the enzymatic convertibility of sugarcane bagasse. Enzyme Microb Technol 2007; 40: 426-32.

[28] Chang VS, Holtzapple MT. Fundamental factors affecting biomass enzymatic reactivity. Appl Biochem Biotechnol 2000; 84-6: 5-37.

[29] Dunlop AP. Furfural Formation and Behavior. Industrial and Engineering Chemistry 1948; 40: 204-9.

[30] Sorensen A, Teller PJ, Lubeck PS, Ahring BK. Onsite Enzyme Production During Bioethanol Production from Biomass: Screening for Suitable Fungal Strains. Appl Biochem Biotechnol 2011; 164: 1058-70.

[31] Carpita NC, Gibeaut DM. Structural Models of Primary-Cell Walls in Flowering Plants - Consistency of Molecular-Structure with the Physical-Properties of the Walls During Growth. Plant Journal 1993; 3: 1-30.

[32] Panagiotou G, Olsson L. Effect of compounds released during pretreatment of wheat straw on microbial growth and enzymatic hydrolysis rates. Biotechnol Bioeng 2007; 96: 250-8.

[33] Xiao ZZ, Zhang X, Gregg DJ, Saddler JN. Effects of sugar inhibition on cellulases and beta-glucosidase during enzymatic hydrolysis of softwood substrates. Appl Biochem Biotechnol 2004; 113: 1115-26.

[34] Garcia-Aparicio MP, Ballesteros I, Gonzalez A, Oliva JM, Ballesteros M, Negro MJ. Effect of inhibitors released during steam-explosion pretreatment of barley straw on enzymatic hydrolysis. Appl Biochem Biotechnol 2006; 129: 278-88.

[35] Ximenes E, Kim Y, Mosier N, Dien B, Ladisch M. Inhibition of cellulases by phenols. Enzyme Microb Technol 2010; 46: 170-6.

[36] Taherzadeh MJ, Niklasson C, Liden G. Acetic acid - friend or foe in anaerobic batch conversion of glucose to ethanol by Saccharomyces cerevisiae?. Chemical Engineering Science 1997; 52: 2653-9.

[37] Delgenes JP, Moletta R, Navarro JM. Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by Saccharomyces cerevisiae, Zymomonas mobilis, Pichia stipitis, and Candida shehatae. Enzyme Microb Technol 1996; 19: 220-5.

[38] Stenberg K, Galbe M, Zacchi G. The influence of lactic acid formation on the simultaneous saccharification and fermentation (SSF) of softwood to ethanol. Enzyme Microb Technol 2000; 26: 71-9.

[39] Luo L, van dV, Huppes G. Biorefining of lignocellulosic feedstock - Technical, economic and environmental considerations. Bioresour Technol 2010; 101: 5023-32.

[40] Garrote G, Dominguez H, Parajo JC. Interpretation of deacetylation and hemicellulose hydrolysis during hydrothermal treatments on the basis of the severity factor. Process Biochemistry 2002; 37: 1067-73.

Paper II

Arvaniti E, Z Benko, K Reczey, AB Bjerre, JE Schmidt, Production of enzyme by *Trichoderma reesei* for hydrolyzing pretreated rape straw, Bioresource technology, 2010, submitted

II PRODUCTION OF ENZYME BY *TRICHODERMA REESEI* FOR HYDROLYZING PRETREATED RAPE STRAW

Contributors: Efthalia Arvaniti, Zsuzsa Benko, Kati Reczey, Anne Belinda Bjerre, Jens Ejbye Schmidt

Abstract

Rape straw is a lignocellulosic agricultural residue that can be used as feedstock for ethanol production in addition to carbon source for production of enzymes for hydrolysis of rape straw for ethanol production. In this study, the best enzyme mixture for hydrolyzing cellulose and hemicellulose of pretreated by Wet oxidation (WO) rape straw are investigated. For this, available commercial enzyme mixtures from Novozymes, as well as bench-scale produced enzymes from *T. reesei* with pretreated rape straw as carbon source are tested. Four fractions of WO pre-treated rape straw: (1) moist filter cake, (2) whole slurry, (3) partly detoxified whole slurry, and (4) partly detoxified filtrate were applied as carbon source for *Trichoderma reesei*. All enzymes are evaluated by the achieved glucose and xylose yields with WO pretreated rape straw. In addition, produced enzymes are evaluated by the achieved enzyme activity yields.

The significantly highest glucose yield was achieved for the commercial enzymes Celluclast and Cellubrix (77-79%). Among the enzymes produced with pretreated rape straw, the highest glucose yield was achieved by enzymes produced with whole slurry ranging 71-72%. The glucose yield was similar to the reference case using Solka floc as carbon source. The highest xylose yield was achieved by the commercial enzymes Celluclast and Cellubrix and by the enzymes produced from produced with Solka floc,

ranging 74-78%, and for enzymes produced with whole slurry xylose yield was 66-74%.

Among the fractions of pretreated rape straw carbon source, whole slurry induced the highest FPA yields 123 FPU/g monosaccharide equivalents (at 11 days). Detoxification of filtrate and whole slurry did not improve enzyme production. Enzymes produced from detoxified whole slurry hydrolyzed 21% less glucose and 11% less xylose than from the equivalent non-detoxified. The β-glycosidase activity was very low in all enzyme mixtures (commercial and produced). As for enzymes produced with whole slurry it was estimated that about 20% of pretreated rape straw filter cake needs to be allocated for enzyme production (carbon source), while the rest can be used for bioethanol production.

1 Introduction

Lignocellulose comprises of crystalline and non crystalline regions of long cellulose fibrils that are interconnected loosely with hemicellulose chains, and both are coated like cement by lignin [1]. Conversion of lingo-cellulosic biomass to 2G bioethanol requires development of an efficient saccharification step for releasing fermentable sugars [2]. Saccharification of carbohydrates from lignocellulose like cellulose can be facilitated by hydrolytic enzymes [1,3]. For achieving complete enzyme saccharification of cellulose, a combination of a digestible lignocellulosic substrate together with an effective enzyme system is needed [4]. Digestibility of lignocellulosic substrates to enzymes is enhanced by a pretreatment step like wet oxidation that removes part of the lignin and hemicellulose to allow enzymes reach and hydrolyze cellulose to monosaccharides [5]. On the other hand, effective cellulolytic enzyme systems are either available in the market or can be produced onsite by enzyme fermentation. The enzymes that hydrolyze cellulose fall into three groups:

1. Endoglucanases (EGs) fragment random accessible β-1,4-glycosidase bonds of (crystalline) cellulose chains at random positions. They decrease the viscosity, and create chain-ends.
2. Cellobiohydrolases (CBHs) remove "processively" units of disaccharides of cellobiose from chain-ends of cellulose.
3. Finally, β-glycosidases (βGs) hydrolyze cellobiose to glucose. βGs are not strictly speaking cellulases, but are essential in the enzyme mixture for complete saccharification of cellulose to glucose.

Synergistic activity of these three groups is necessary for complete saccharification of cellulose to glucose [6].

Trichoderma reesei is the most well studied and industrially applied cellulase producing microorganism, and particularly Celluclast 1.5L and Cellubrix L are enzyme products of *T. reesei* branded by Novozymes [7]. *T. reesei* is an aerobic filamentous fungus *that* grows preferably on glucose and xylose [8,9], and produces and secretes two CBHs comprising 75% and a number of EGs approx 20% of total secreted protein. The mechanism is triggered by inducing, and end-product inhibition systems, which are not fully understood yet, although full genome of *T. reesei* has been mapped [10-13]. It is known that although *T. reesei* is superior in producing EGs and CBHs, the produced βG is bound to the mycelium, and difficult to recover [13,14]. Therefore, for complete saccharification of cellulose to glucose, βG needs to be dosed exogenously, by for example *Aspergillus niger*. βG from *A. niger* is also branded as Novozym188 by Novozymes and traditionally supplements Celluclast 1.5L in enzyme applications. Finally, in addition to the above three groups of cellulases, *T. reesei* produces 30 other minor proteins consisting of hemicellulases and other accessory proteins [13] that enhance the digestibility of cellulose by saccharifying hemicellulose that covers cellulose [15].

The composition of the enzyme mixture and the quantity of the produced enzymes from *T. reesei* Rut C-30 is dependent on the carbon source and its composition [11,12,16,16,17]. This dependency is to such an extent that it has been suggested that the achieved sugar yields from enzymatic saccharification of lignocellulosic substrate are highest, when the carbon

source for enzyme production is of the same lignocellulosic material [11,18]. Because of the possible correlation between carbon source of *Trichoderma* and the composition of the produced enzyme cocktail, many complex carbon sources have been tested for production of cellulases [8,16,19,20].

Rape straw is an abundant low cost agricultural residue that has similarities to wheat straw [21] and has been suggested for ethanol production [22]. However, to the best of our knowledge it has not been investigated which enzyme mixture is the best for hydrolyzing rape straw, after it has been pretreated by wet oxidation. Moreover, rape straw has never been examined as carbon source for production of cellulases.

In this study, appropriate enzymes for hydrolyzing WO pretreated rape straw are sought. among available commercial enzymes mixtures, and produced mixtures by *T. reesei* with pretreated rape straw as carbon source. In enzyme producing experiments, four fractions of WO pre-treated rape straw that is 1) moist filter cake, 2) whole slurry, 3) partly detoxified whole slurry, and 4) partly detoxified filtrate were applied as carbon sources and inducers for *T. reesei*. Subsequently, the enzymes produced are compared with commercial enzymes for their effectiveness in hydrolyzing cellulose from pretreated rape straw, measured as glucose yields. Secondly, the enzymes are evaluated on their capacity to hydrolyze insoluble hemicellulose of pretreated rape straw, measured as the xylose yield. And finally, the enzyme production efficiency is evaluated based on the achieved enzyme activity yields per gram of added monosaccharide equivalents.

2 Materials and methods

2.1 Carbon sources and substrates

Solka floc 200 and rape straw were used as carbon sources for enzyme production. Solka floc 200 is delignified wood cellulose and was used as

reference carbon source in enzyme production experiments. It was purchased by International Fiber Europe NY, USA.

Pretreated rape straw was used both as carbon source for enzyme production experiments, and as substrate of hydrolytic enzymes for production of fermentable sugars. Rape straw was collected by the fields around Risø DTU campus in Roskilde Denmark at the end of the summer of 2007, and was first air-dried to 90-95% humidity and stored at room temperature. Before use the straw was milled by knife mill to 2 mm particle size and pre-treated by wet oxidation (WO) in a 2 L loop reactor at 6% dry matter (DM) content with 12 bar of oxygen gas pressure at 195 ^{o}C for 15 minutes as described earlier [22], but without addition of extra chemicals. The produced pretreated slurry was separated by vacuum filtration with 0.1 mm mesh nylon filter into moist filter cake (about 17.2% DM) and filtrate and both were stored at -20 ^{o}C. Upon use, both filter cake and filtrate were defrozen, and either used alone or mixed again with the filtrate in original ratios (%wt.). Also, part of the filtrate was concentrated 3-fold by vacuum evaporation (Heidolph, Laborotta 4000 Efficient) at 55^{o}C to reduce contained volatile compounds; therefore there were one filter cake and two qualities of filtrates.

2.2 Inoculum and enzyme production in shake flasks

Stock culture: *Trichoderma reesei Rut C 30* stock cultures were cultured on sterilized (121 ^{o}C, 20 minutes) malt agar slants containing: 30g/l malt extract, 5 g/l glucose, 1 g/l proteose peptone, and 20 g/l bacto agar at pH 6.2 and 30 ^{o}C for two weeks.

Inoculum: The greenish conidia of *T. reesei* were grown in two agar slants for 2 weeks and were suspended in 5 ml sterile water each, and 1.5 ml were transferred to 750 ml Erlenmeyer flask, containing 150 ml sterile modified Mandel's medium [23]. Modified Mandel's medium consisted of 1.87 g/l $(NH_4)_2SO_4$, 2.67 g/l KH_2PO_4, 0.53 g/l $CaCl_2$, 0.40 g/ml carbamid, 0.81 g/l $MgSO_4.7H_2O$, 0.33 g/l yeast extract, 1.00 g/l proteose peptone, and 10 g/l monosaccharide equivalents of Solka Floc 200, and pH was adjusted to 5.8. The medium was supplemented with metal trace elements, 6.6 ppm

$FeSO_4.7H_2O$, 26.7 ppm, $CoCl_2$, 2.1 ppm $MnSO_4$, and 1.9 ppm $ZnSO_4$. Incubation was done in triplicates for 4 days at 30 °C shaken at 350rpm, before start of enzyme fermentation.

Fermentation media: The fermentation medium contained modified Mandel's medium that consisted of 0.4 g/l carbamid, 1.87 g/l $(NH_4)_2SO_4$, KH_2PO_4 2.67 g/l, $CaCl_2$, 0.53 g/l, $MgSO_4.7H_2O$ 0.81 g/l, yeast extract 0.33 g/l, proteose peptone 1 g/l, and the following trace elements: $FeSO_4$ 0.66ppm, $MnSO_4$. H_2O 0.21 ppm, $CoCl_2$ 2.67 ppm [23]. The medium was supplemented by 0.1 M Tris-maleate buffer with 11.6 g maleic acid and 12.11 g Tris-(hydroxymethyl)aminomethane, and adjusted to pH 6.

Five carbon sources were tested in enzyme production experiments, four of which were fractions of WO pretreated rape straw and finally Solka floc, serving as reference substrate. Table 1 describes the carbon sources, together with their acronym.

The media of 150 ml final volume were sterilized at 121°C, for 20 minutes before inoculation.

Table 1 ***D*****escription of carbon sources**

Description of carbon source
Reference experiment; Solka floc 200
Slurry from pre-treated rape straw
Slurry from pre-treated rape straw, containing partly detoxified filtrate
Moist filter cake of pre-treated rape straw
Concentrated partly detoxified filtrate

Fermentation: 10% v/v of inoculum was added under sterile conditions to the 150 ml fermentation medium in triplicates. After inoculation, shake flasks were agitated at 350 rpm at 30 °C for 11 days. Every two-three days, samples were taken from the bottles under sterile conditions and pH was readjusted to 6 (±0.2) if necessary with NaOH 2 M and HCl 2 M. After the end of the fermentation, all fermentation broths were centrifuged and the enzyme rich

supernatants were separated from solids and stored in a freezer at -20 °C for further analysis.

2.3 Enzyme assays

All samples taken during enzyme fermentation were analyzed for reducing sugars, cellulase activity, and βG activity. Reducing sugars were measured with the 3,5-dinitro-salicylic acid (DNS) method [24], βG activity via Berghem method [25], and cellulase activity by Filter-Paper Activity (FPA) via Mandel's method [26].

2.4 Enzymatic saccharification

The produced enzyme mixtures were tested in hydrolyzing filter-cake of WO pre-treated rape straw substrate, and compared to commercial available enzymes. The list of enzyme mixtures tested is presented in Table 2. Before use, substrates and enzymes were adjusted to pH 4.8. Substrate was added at 2% DM loading, and enzyme mixtures were supplemented with 100 IU/g DM commercial βG (Novozym 188, Novozymes) to a total volume of 8 ml. Enzyme assays were rotary shaken in triplicate at 50 °C for 24 hours, and they were in triplicates and with an enzyme blank. Cellubrix L (with FPA 75 FPU/ml and βG activity was 33 IU/ml), Celluclast 1.5 L (with FPA 83 FPU/ml and βG activity 32 IU/ml) and Novozym 188 (with βG activity 408 IU/ml) were gifts from Novozymes (Bagsværd, Denmark).

Table 2 List of enzyme mixtures used at enzymatic saccharification experiments of pretreated rape straw. All enzyme mixtures were supplemented with Novozym 188 at 100 IU/g DM βG activity

Cellulases origin	*Dosage of cellulases (FPU/g DM)*
Cellulases produced with Solka floc carbon source	30
Cellulases produced with Slurry carbon source	30
Cellulases produced with Detoxified Slurry carbon source	30
Cellulases produced with Filter cake carbon source	30
Cellubrix L	30
Celluclast 1.5L	25

2.5 Chemical analysis

Solids analysis: The raw material and the filter-cake after pre-treatment were dried at 105 ^{o}C and analyzed for total sugars (glucose, xylose, and arabinose) through strong acid hydrolysis and Klason lignin, and ash as mentioned earlier [22].

Liquids analysis: The filtrate after pre-treatment was analyzed for glucose, xylose, and arabinose oligosaccharides through weat acid hydrolysis and monosaccharides as reported earlier [27], and common enzyme and yeast inhibitors like organic acids (acetic acid, formic acid), and furfural by HPLC.

Also, after enzymatic saccharification experiments, samples were centrifuged and supernatants were analyzed for simple sugars (glucose, cellobiose, xylose, and arabinose) with HPLC.

The HPLC (Shimadzu Corp, Kyoto, Japan) was equipped with a CDD-10 RI-detector for sugars and acids, and a UV-detector for furans, Shimadzu Corp, Kyoto, Japan), and used H_2SO_4 4 mM as eluent.

2.6 Calculations

Sugar and sugars equivalents were calculated from HPLC data in the hydrolyzed form as monosaccharides, thereby multiplying all polysaccharides and oligosaccharides with their hydration factor. For cellulose the hydration factor (H_f) is 1.1, and for hemicellulose (arabinoxylan) it is 1.1.36.$_f$

Enzyme titter is expressed as **Filter-paper activity** (FPA) and was calculated by equation 1:

$$FPA\ (\frac{FPU}{ml}) = \frac{Abs_{550} - b}{m} \times \frac{1000 \times D}{V \times M \times t} \quad \text{(eq1)}$$

Where Abs_{550} is the absorbance at 550 nm (-), b is the intercept of DNS calibration (-), m is the slope of DNS calibration (mol min^{-1} g^{-1} FPU^{-1}), V is the volume of the sample (0.5 ml), M is the molecular weight of glucose (180 g mol^{-1}), t is the reaction time (60 min), and D is the dilution rate before the assay (-).

FPA yield is expressed as FPU per gram of added monosaccharide equivalents at time zero of each carbon source in the enzyme fermentation experiments.

$$FPA\ yield\ \left(\frac{FPU}{g\ total\ sugars}\right) = \frac{FPA\ activity}{added\ total\ monosaccharide\ equivalents} \quad \text{(eq 2)}$$

Where enzyme titter is the measured FPA, and the denominator contains the total amount of monosaccharide equivalents from each carbon source was added in enzyme fermentation at timer zero.

To highlight the use of filter cake in enzyme production experiment with slurry,

FPA yield is exclusively expressed also as FPU per gram added filter cake (g dry pretreated insoluble biomass) in enzyme production experiments and is based on the FPA yield 1 value for SLU.

$$FPA\ yield\ \left(\frac{FPU}{g\ dry\ filter\ cake}\right) = \frac{FPA\ yield \times H_f \times C_{C/H}}{R_{sFC/SLU}} \quad \text{(eq.3)}$$

Where FPA yield 1 is the yield based on monosaccharides equivalents presented above, H_f is the hydration factor of carbohydrates and for cellulose is 1.1, $C_{C/H}$ is the concentration of carbohydrates in pretreated filter cake, and $R_{sFilter\ cake}$ is the ratio of mass of sugars in filter cake (Filter cake) over the mass of sugars present in slurry (SLU) carbon sources.

Total **enzyme productivity** is calculated based on Filter-paper activity by equation:

$$\text{FPA productivity (FPU } L^{-1}h^{-1}) = \frac{Filter\ Paper\ activity \times 1000}{duration\ of\ fermentation\ in\ hours} \quad \text{(eq. 4)}$$

βG activity was calculated based on equation 3:

$$\beta G\ activity\ \left(\frac{IU}{ml}\right) = \frac{Abs_{400}}{m} \times D \quad \text{(eq.5)}$$

where Abs_{400} is the absorbance at 400 nm (-), m is the slope of the calibration curve (ml IU^{-1}), and D is the dilution of the enzyme solution before analysis (-).

Glucose yield % in enzymatic hydrolysis of cellulose was calculated in a percentage basis of the glucose potential of added pretreated biomass (100%):

$$\text{Glucose yield } \left(\frac{g \text{ released } glucose\ after\ enzyme\ hydrolysis}{\text{g glucose potential of added in enzyme hydrolysis}}\right) = \frac{C_{Gl}}{C_{Cel} \cdot H_f} \cdot 100\% \qquad \text{(eq. 6)}$$

C_{Gl} is the released glucose in enzyme hydrolysis experiments $(\frac{g}{l})$, C_{Cel} is the concentration of cellulose added in enzyme hydrolysis experiments $(\frac{g}{l})$, and H_f is the hydration factor of glucose $(\frac{g\ glucose}{g\ cellulose})$ equal to 1.1.

Under the same principle, **xylose yield %** was calculated, as the percentage of the released xylose after enzyme hydrolysis of the xylose potential hemicellulose (xylan). For xylose H_f is 1.136.

Standard deviation was used for analyzing the dispersion of duplicate experiments, and stems all graphic illustrations in figures. The standard deviation was produced using STDEVA formula of Excel software for an array of data that cumulatively normalized in a single average value.

One-way analysis of variance analyzed data for 5% significance level by of grouped experiments (treatments) with different conditions (levels). For example, enzyme titter results from samples of day seven were analyzed together. In the case, the group analyzed with ANOVA contained more than two levels, and results were found significantly different by ANOVA, **Newman and Keuls statistical analysis** tool was applied at 5% significance level, to identify in which levels of the treatment were significantly different. Newman and Keuls was calculated with DSAASTAT macro v. 1.101 (Perugia, Italy) in Excel software. **Correlation coefficient** was applied between two arrays of variables for determining their relationship. Here is used to relate either a dependent variable to an independent variable, or two dependent variables. Correlation coefficient was calculated by a model from Excel software Microsoft office 2007.

3 Results

3.1 Enzyme production

The composition of carbon sources added in enzyme producing experiments in listed in Table 3. Solka floc (reference carbon source) contained only 82% cellulose (glucan) and 17% hemicellulose (xylan). Filter cake is very similar to the composition of Solka floc but contained except polymeric sugars, also other components like solid lignin, and oligomeric sugars, organic acids, furans and phenols among others. Slurry contained more soluble components than filter cake, and fewer polymers. Slurry containing filtrate that was partly detoxified (now Detoxified Slurry) had similar composition as non-detoxified Slurry (now Slurry), but contained less soluble volatile compounds like organic acids, furans and phenols. HMF was not detected (data not shown). Finally the filtrate that was partly detoxified (now Detoxified Filtrate) contained only soluble compounds and principally oligomeric sugars, and again had limited content of volatile compounds. Although Detoxified Filtrate was concentrated by evaporation, contained lower total sugars (11.5 g/l) than the other carbon sources (15.4-17.3 g/l) due to caramelization of sugars in evaporation glass walls (data not shown).

Comparison of Slurries (with and without Detoxification) in Table 3 shows that controlled evaporation of filtrate before use reduced concentration of volatile compounds (see Detoxified Slurry) like formic acid, acetic acid, or furfural, and phenolics; formic acid and acetic acid was reduced more than 2-fold, furfural more than 13-fold and halved phenolics.

The produced enzyme titters in terms of FPA are presented in Figure 1. Results of enzyme titter at sampling days 4, 7, 9, and 11 were found significantly different for all 4 tested days. The FPA of Solka floc was significantly higher among all carbon sources for day 7 and day 11 (see Figure 1). The significantly highest enzyme titter for carbon sources derived from pretreated rape straw was recorded for enzymes produced with Filter cake as carbon source at 7 days 2.0 FPU/ml, and for enzymes produced with slurry (Slurry) at 11 days 1.9 FPU/ml. The FPAs of enzymes produced with Slurries as carbon source were not significantly different for day 7, but in day 11 the FPA of enzymes produced with Detoxified Slurry as carbon source was lower (1.6

FPU/ml) than that produced with Slurry. Finally, the significantly lowest FPA was recorded for enzymes produced with Detoxified Filtrate, with utmost 0.6 FPU/ml after 14 days, which makes partly detoxified Filtrate the least inductive carbon source of all tested. The results from Newman-Keuls post-hoc statistical tool are summarized in Table 4 and Table 5. As can be seen in Table 4but also in Figure 1, all experiments had a decrease of FPA at day 9, which resulted in producing non-significantly different FPAs for enzymes produced with Solka floc, Filter cake, Slurry, or Detoxified Slurry. For experiment with Filter cake, as well as Solka floc the significantly highest enzyme titter was achieved at day 7, whereas for both Slurries and Detoxified filtrate the significant maxima were measured for day 11. Beyond these dates (to day 14, data not shown) no significant improvement of FPA was observed for each carbon source.

Table 3 Composition of carbon sources in monosaccharide equivalents, lignin, and main degradation product of WO pretreatment. These carbon sources were used in the media for the enzyme producing experiments. All quantities of sugars calculated as monosaccharide equivalents. NA not analyzed; ND not detected

	Solka floc (g/l)	*Slurry (g/l)*	*Detoxified slurry (g/l)*	*Filter cake (g/l)*	*Detoxified filtrate (g/l)*
Total monosaccharide equivalents	16.9	15.4	15.9	17.4	11.4
Cellulose	13.8	10.8	10.8	14.6	ND
Hemicellulose	3.0	1.3	1.3	1.8	ND
Glucose	NA	0.1	0.1	ND	0.3
Xylose	NA	0.6	0.5	0.2	1.6
Oligomeric glucose	NA	0.4	0.6	0.2	1.8
Oligomeric C5 sugars	NA	2.1	2.5	0.6	7.7
Lignin	ND	3,3	3,3	4,5	NA
Formic acid	NA	0.6	0.3	0.2	1.0
Acetic acid	NA	0.8	0.4	0.2	1.3
Furfural	NA	0.12	0.01	0.03	0.03
Total Phenolics	NA	0.48	0.47	0.14	1.4

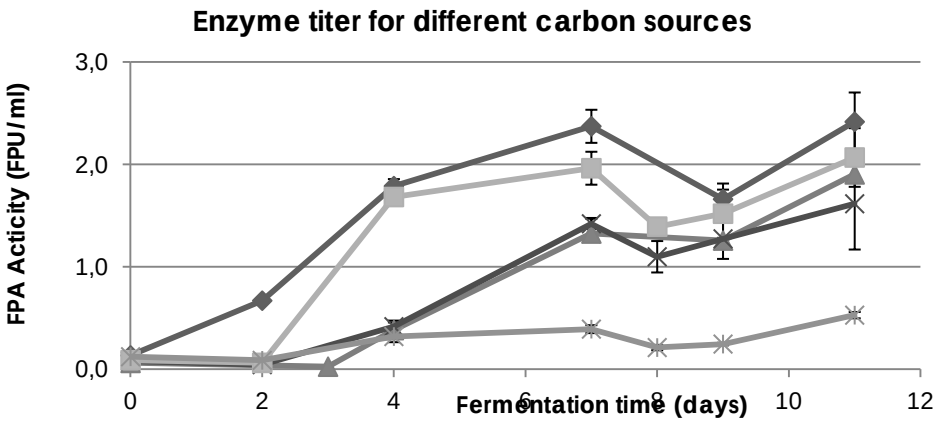

Figure 1 Enzyme titter (FPU/ml) of fermentation of different carbon sources over time: Solka floc, reference (rhomb); Slurry (triangle); Detoxified Slurry (X symbol); Filter cake (square); Detoxified Filtrate (star). The error bars show the standard deviations of the triplicates.

Table 4 Data from Newman-Keuls analysis at 95% confidence of the enzyme titters (FPU/ml) achieved with 5 carbon sources. According to ANOVA, the enzyme titters for all tested five carbon sources were significantly different in all tested days 4, 7, 9, and 11, and here are ranked from a to d with a the highest, and d the lowest.

Sampling day	Carbon sources				
	Solka floc	Slurry	Detoxified Slurry	Filter cake	Detoxified Filtrate
Day 4	a	c	c	b	d
Day 7	a	a	a	a	b
Day 9	a	b	c	b	d
Day 11	a	c	c	b	d

Table 5 Data from Newman-Keuls analysis at 95% confidence of the enzyme titters (FPU/ml) achieved for each carbon source at each sampling day. According to ANOVA, the enzyme titters for all tested sampling days were significantly different in all tested carbon sources, and here are ranked from a to d with a the highest, and d the lowest.

Carbon source	Sampling days			
	4	7	9	11
Solka floc	b	a	c	a
Slurry	c	b	b	a
Detoxified Slurry	c	ab	b	a
Filter cake	b	a	c	a
Detoxified Filtrate	d	b	c	a

Figure 2 shows the FPA productivities (FPU $L^{-1}h^{-1}$) and revealed that all experiments with carbon sources derived from pretreated rape straw had a lag-phase of one to three days, comparing to the reference carbon source experiment (Solka floc). After 4 days of fermentation, the FPA productivities of enzymes produced with Filter cake and Solka floc were not significantly different (see Table 4) and their productivity was maximum ranging from 17.5-18.6 FPU $L^{-1}h^{-1}$. However after 7 days the FPA productivities of enzymes produced in Filter cake and Solka floc significantly decreased by 33% and 24% respectively. On the contrary for the FPA productivities of enzymes produced with Slurries after 4 days were not significantly different ranging from 4.0-4.3 FPU $L^{-1}h^{-1}$ respectively, and after 7 days they almost doubled for both. Finally, FPA productivity of enzymes produced with Detoxified Slurry was 2-3 FPU $L^{-1}h^{-1}$ throughout the duration of the fermentation.

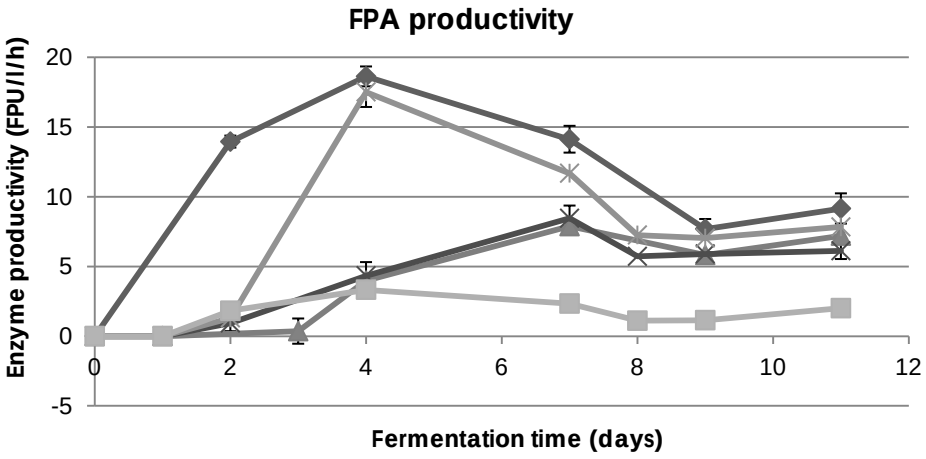

Figure 2 FPA productivity for two sampling days for Enzymes on different carbon sources: Solka floc, reference (rhomb); Slurry (triangle); Detoxified Slurry (X symbol); Filter cake (square); Detoxified Filtrate (star). The error bars show the standard deviations of the triplicates.

Because the amount of added monosaccharide equivalents were not the same for each carbon sources (see Table 3), FPA yields per gram of added monosaccharides were calculated to normalize the differences in Figure 3. The FPA yields for day 4, 7, 9, and 11 were found significantly different (see Table 6). The FPA yield that exhibited the significant maximum was with Solka floc at 140 FPU/g added monosaccharide equivalents at 7 days of fermentation. Maximum FPA yield among the carbon sources derived from

pretreated rape straw was for enzymes produced on Filter cake 113 FPU/ g added monosaccharide equivalents at 7 days and for enzymes produced with Slurry 123 FPU/ml at 11 days of fermentation. Beyond these days according to Table 5, no further improvement was observed for enzyme titter, which is valid for FPA yields. FPA yields of enzymes produced with Slurries were not significantly different for days 4, 7, and 9, but at day *11* FPA yield of enzymes produced with Slurry significantly increased, compared to partly detoxified Slurry (102 FPU/g *added* monosaccharide equivalents*)*. Finally, the lowest FPA yield was observed for enzymes produced on filtrate (Detoxified Filtrate) 46 FPU/g added monosaccharide equivalents after 11 days.

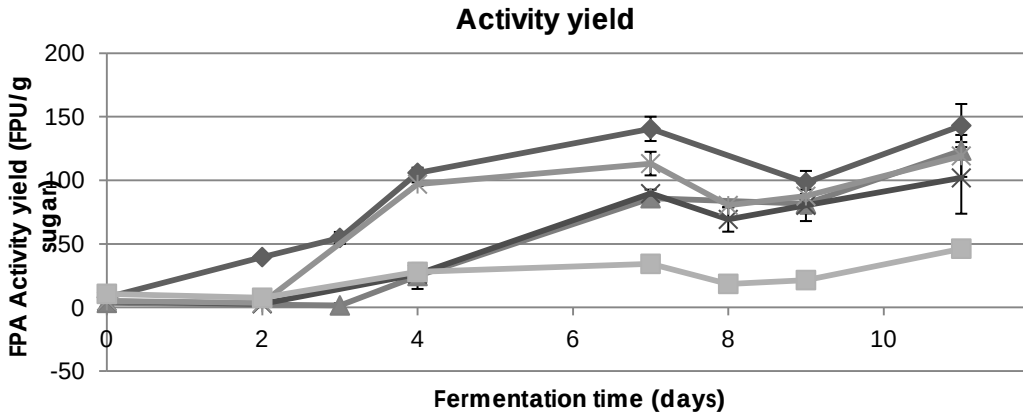

Figure 3 Enzyme FPA yields for enzymes produced with different carbon sources: SF, reference (rhomb); Solka floc, reference (rhomb); Slurry (triangle); Detoxified Slurry (X symbol); Filter cake (square); Detoxified Filtrate (star). The error bars show the standard deviations of the triplicates.

Table 6 Data from Newman-Keuls analysis at 95% confidence of the FPA yields (FPU/g added monosaccharide equivalents) achieved at three sampling days with 5 carbon sources. The FPA yields according to ANOVA were significantly different for days 4, 7, 9, and 11, and here are ranked from a to d with a the highest, and d the lowest value.

	Carbon sources				
	SF	SLU	Detoxified Slurry	Filter cake	Detoxified Filtrate
Day 4	a	c	c	b	c
Day 7	a	c	c	b	d
Day 9	a	b	b	b	c
Day 11	a	b	c	b	d

From Table 3 before, it was observed that both Slurries and Detoxified Filtrate carbon sources contained the highest amount of soluble sugars. It can be seen that evaporation of volatile components of the filtrate of Detoxified Slurry resulted in removal of 46% of formic acid, 45% of acetic acid, and 91% of furfural.

The concentration of reducing sugars over time during enzyme production is illustrated in Figure 4 and the statistical results are presented in Table 7. All concentrations of reducing sugars for days 4, 7, 9, and 11 were significantly different except at day 7, where only reducing of partly detoxified Filtrate were significantly different. Concentration of reducing sugars of partly detoxified Filtrate at time zero of Figure 4 was the highest among the experiments and decreased below 1.4 g/l after 9 days. The concentration of reducing sugars for all other experiments decreased below 0.85 g/l after only 6 days.

Together with FPA, which measured the activity of cellulases, the βG activity was also measured every two days and results are presented in Figure 5. Although results were found significant, βG activity for all experiments including the reference activity was below 0.1 IU/ml (data not shown).

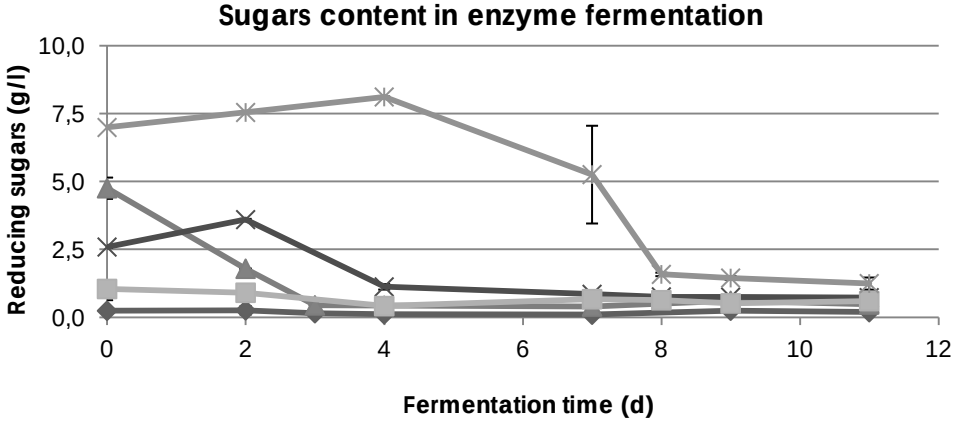

Figure 4 Concentration of reducing sugars in g/l of medium during enzyme fermentation experiments with different carbon sources: Solka floc, reference (rhomb); Slurry (triangle); Detoxified Slurry (X symbol); Filter cake (square); Detoxified Filtrate (star). The error bars show the standard deviations of the triplicates.

Table 7 Data from Newman-Keuls analysis at 95% confidence of the reducing sugars (g/l) achieved at three sampling days with 5 carbon sources. The FPA yields according to ANOVA were significantly different for days 4, 7, 9, and 11, and here are ranked from a to d with a the highest, and d the lowest value.

	Carbon sources				
	SF	SLU	Detoxified Slurry	Filter cake	Detoxified Filtrate
Day 4	e	c	b	d	a
Day 7	b	b	b	b	a
Day 9	e	c	b	d	a
Day 11	e	d	b	c	a

3.2 Enzyme testing

Harvested and commercial cellulolytic enzymes (see Table 2) were tested on enzyme assays on their capacity to hydrolyze pre-treated rape straw substrate and compared. For this, all cellulase mixtures were first supplemented by Novozym 188 to secure sufficient βG activity.

The results from the 24 hours enzyme saccharification experiments expressed in glucose yields and xylose yields were significantly different and are shown in Figure 5The highest glucose yield was observed for commercial cellulases Celluclast and Cellubrix that were not significantly different (data not shown), ranging from 78-80%. The difference in glucose yields between the commercial enzymes was not significant, but Celluclast was dosed at 25 FPU/g DM compared to Cellubrix that was dosed at 30 DM/g DM. Glucose yields from produced enzymes were highest for enzymes produced with Slurry and with Solka floc (reference carbon source) that were not significantly different ranging 71-72%. Finally, the glucose yields of enzymes produced with Detoxified Slurry and with Filter cake were not significantly different ranging from 54-56%.

Regarding hemicellulose (xylan) saccharification, highest achieved xylose yield was for Cellubrix and enzymes produced with Solka floc that were not significantly different ranging from 75-78%. The next best xylose yields were achieved with enzymes produced with Slurry and Celluclast enzyme mixtures that were not significantly different and ranging from 66-74%. Finally, the last two enzyme mixture was enzymes produced with Filer cake and with

Detoxified Slurry that were not significantly different and ranging from 53-58%. During the WO pre-treatment of rape straw, 68% of hemicellulose (arabinoxylan) was removed (data not shown), and only 24% was insoluble in pretreated rape straw filter cake, and comprised solely of xylose (xylan). The above xylose yields were calculated based on the xylose potential of the insoluble hemicellulose of pretreated rape straw filter cake. The correlation coefficient between glucose yields and xylose yields was 0.83.

In Slurry as shown in Table 3, 79% of the sugars and monosaccharide equivalents originated from filter cake and 21% from the filtrate of pretreated rape straw (see Table 3). The FPA yield of enzymes produced with Slurry (123 FPU/g sugar equivalents) can be also expressed in FPU per gram of filter cake spent, according to (eq.3). Given that after pretreatment moist filter cake contains 69.5% cellulose and hemicellulose (data not shown), the FPA yield would be 120 FPU/*g* filler cake (dry matter, DM).

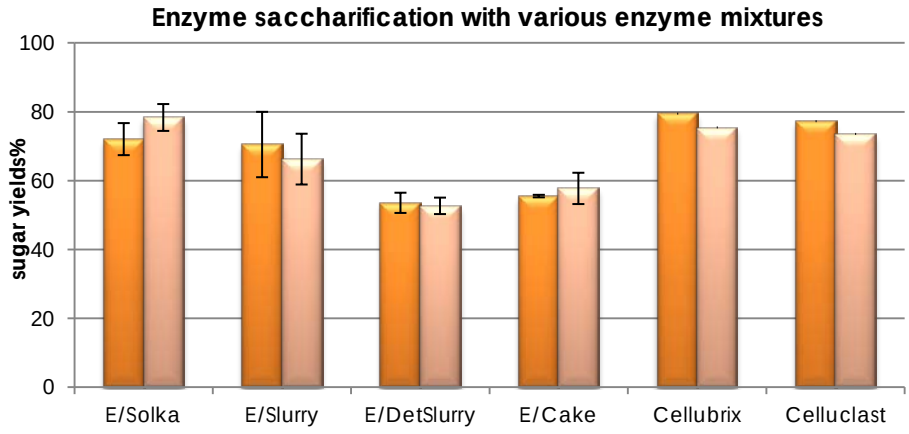

Figure 5 Sugar yields of glucose (orange), and xylose (pink), based on sugar potential of added substrate (WO pretreated rape straw filter cake), after 24 hours enzymatic hydrolysis with different enzyme mixtures; E/Solka) contains enzymes produced on Solka floc; E/Slurry) contains enzymes produced on Slurry; E/DetSlurry) contains enzymes produced on Detoxified Slurry; E/Cake) contains enzymes produced on Filter cake; Cellubrix) contains Cellubrix enzymes; Celluclast) contains Celluclast enzymes. All enzyme mixtures contain also Novozym 188. The error bars give the standard deviation of the enzyme assays containing produced enzymes.

4 Discussion

The aim of the study was to find and evaluate some of the available cellulolytic enzymes for hydrolyzing WO pretreated rape straw into glucose. Both produced enzymes as well as commercial enzyme formulations were tested. Enzymes were produced by *Trichoderma reesei* using fractions of WO pretreated rape straw as carbon sources. Four fractions were tested: moist filter cake, whole slurry, whole slurry containing filtrate that was partly detoxified by evaporation, and finally partly detoxified filtrate alone. As reference carbon source in enzyme production experiments Solka floc was used. The efficiency of the enzymes were assessed by the produced glucose yields in enzyme assays with pretreated rape straw substrate, and to a lesser extent by the achieved xylose yields with same substrate. Finally, production efficiency of enzymes from four carbon sources was evaluated on enzyme yields.

Because all enzymes (both produced and commercial) were found deficient in β-glycosidase (βG) activity For this before tested in enzyme assays for hydrolyzing pretreated rape straw substrate, they were dozed with equal amount of Novozym188 100 IU/g DM. The significantly highest glucose yields in 24 hours enzyme assays were achieved with commercial cellulase formulation Celluclast and Cellubrix. The glucose yields were 10% higher than the glucose yield from enzymes produced using Solka floc or (non-detoxified) whole slurry as carbon source. Comparing glucose yields of produced enzymes with commercial cellulolytic formulations with the same substrate was interesting given that in all cases *T. reesei* was the host strain, but production conditions were different, which supports the hypothesis that fermentation process conditions reflect in cell growth and expression of certain enzymes in the enzyme mixture [28,29].

The production method of Celluclast and Cellubrix is patented for many decades [7]. From a Novozymes patent [30], it can be deducted that Celluclast and Cellubrix formulations were produced at industrial scale with *Trichoderma reesei* strains with solid lignocellulosic carbon source. Additionally, they were purified and stabilized before reaching the market shelf. More specific information for applied experimental setup cannot be retrieved. Contrary to that setup, hereby produced enzymes were produced

with bench scale shake flask experiments with daily control of pH and passive oxygenation. Despite of the differences of the experimental setup and process conditions mentioned above, the fact that glucose yields of enzymes produced with whole slurry were not significantly different to glucose yields obtained from enzymes produced with Solka floc, indicated the potential of using of pretreated rape straw as carbon source, and further study is required.

Remarkably, enzymes produced with filter cake achieved much lower glucose yields (54%) than those with slurry (71%). Therefore, use of filtrate in enzyme production appears to have improved enzyme capacity to hydrolyzed pretreated rape straw filter cake substrate. The enzyme mixture containing enzymes produced with filter cake achieved the lowest glucose yield together with enzymes produced with partly detoxified slurry. The reason behind this improvement could not be explained based on obtained data. however obtained glucose yields from applying enzymes on the same substrate and under the same conditions, indicated that each carbon source induced different production and secretion of enzymes in *Trichoderma*, and therefore altered the effectiveness of the produced enzyme mixture [31,32].

The effect of evaporating part of the filtrate in the carbon source had an impact in the glucose yields achieved by the respective produced enzymes mixtures. The glucose yields of enzymes produced with slurry with detoxified filtrate were significantly lower (21%) from those produced with non-detoxified slurry. Since all other conditions were kept the same, in enzyme production setup, these results imply that volatile components present in filtrate induced enzyme production that improved the achieved glucose yields with pretreated rape straw. From all known inducers for *T. reesei* found in literature [6,33-35], none of them identified as volatile though.

Regarding obtained xylose yields from hydrolyzing hemicellulose of pretreated rape straw, xylose yields for all experiments generally correlated with the obtained glucose yields (see Figure 4) with a correlation coefficient of 0.83. Removal of hemicellulose has been suggested to uncover cellulose, and make it more accessible to enzymes [36]. Among all tested enzymes, enzymes produced with Solka floc scored the significantly highest xylose yields together with Cellubrix enzymes. However, since Celluclast was dosed

lower than all other enzymes because in the lab Celluclast is famous for being more powerful than Cellubrix but this did not allow straight forward comparison. Nevertheless, the xylose yields show that Celluclast and Cellubrix are more likely comparable. In our experiments hemicellulase activity of enzymes (both produced and commercial) was not analyzed, and enzymes were dosed in enzyme assays based on their FPA. Xylose yields significantly increased (see Figure 5) in the order of enzymes produced with: filter cake < slurry < Solka floc. Also, the insoluble hemicellulose content of carbon sources (see Table 3) in enzyme production experiments increased in the order Slurry < Filter cake < Solka floc. Therefore, there could be a correlation between solid hemicellulose content of carbon source and xylose yields in Solka floc. Juhazs et al. [18] also reported that the level of xylan (insoluble polymeric xylose) in the carbon source directly affected production of hemicellulases.

During enzyme production experiments, the carbon sources were first autoclaved before inoculation for sterilizing, and this probably caused some degradation of soluble sugars and proteins by Maillard reaction and also further degradation of other soluble compounds present in soluble lignocellulosic carbon sources [37], like sugars, furans, etc. Autoclaving therefore, is expected to detoxify the pretreated rape straw carbon source. The impact of autoclaving media was not assessed. However, it was decided to autoclave in order to sustain a sterile environment for *Trichoderma*. In industrial scale though, autoclaving will be done by pretreatment of biomass at high temperature and high pressure like in WO.

Solka floc is a model carbon source for *T. reesei*, since it is a material enriched in cellulose that is a strong inducer of enzyme production with Trichoderma and facilitates release of glucose that is appropriate for growth [6,23]. Also Solka floc is a purified material that does not contain lignin or other components that lower the quality of the carbon source [16]. The main drawback of Solka floc in enzyme production is the high purchasing cost (1,500 euro/ton, personal communication 2011 International Fiber Europe, Belgium), and for this alternative carbon source is sought here.

A first observation for the lag-phase in FPA productivities (see Figure 2) with the two Slurries (Slurry and Detoxified Slurry) was that seven days lag-phase

coincided with relatively high concentration of soluble sugars in the medium of Slurry (see Table 3) as has been reported earlier by Szengyel et al [38]. Increase of use of filtrate in carbon sources was in the order Solka floc < Filter cake < Slurry ≈ Detoxified Slurry < Detoxified Filtrate and corresponded to an increase of soluble sugars (mainly C5 oligosaccharides and monosaccharides, see Table 3), and simultaneous increase of other soluble compounds like organic acids, furans and phenols that are present in the filtrate. However *Trichoderma reesei* Rut C-30 is a mutant strain that facilitates enzyme production as long as there are appropriate inducers present in the medium [6,39-41], and is not subjected to catabolite repression by monosaccharides (glucose, xylose) like the wild type (*Trichoderma viride* QM6a, [42]. For this, it is believed that the observed lag-phase in enzyme production with slurries (Slurry and Detoxified Slurry) was caused by the non-sugar compounds present in the filtrate. Low molecular weight organic acid, furans, and phenolic compounds are common degradation products of lignocellulose formed during pretreatment [43], and are popular inhibitors for yeasts and bacteria [9,37,44-49].

In an attempt to verify source of inhibition in enzyme production, two slurries containing same amount of filtrate (Slurry and Detoxified Slurry), of which one filtrate was subjected to a detoxification pre-step by evaporation (partly detoxified Slurry slurry), were compared as carbon sources. From Table 3 it appears that composition of volatile compounds of slurry containing evaporated filtrate is halved in low molecular weight organic acids (formic acid and acetic acid) and furfural is almost removed by evaporation. The only group of potent inhibitors that was not affected by evaporation is the phenolic compounds. Results of enzyme production showed that the two slurries had no significantly difference in FPA productivity and FPA yield until day 9 (see Figure 2), and only at day 11 FPA productivity of non-detoxified slurry (Slurry) increased resulting in significantly higher enzyme titter and FPA yield compare to detoxified slurry. Also, Rumbold et al. [9] and colleagues reported that the LD_{50} values of acetic acid and furfural for *Trichoderma reesei* were 1 g/l for furfural and 20 g/l for acetic acid, which is much higher than obtained results for slurry (see Table 3). Therefore, it is more likely that inhibition had different origin. As a result it appears that removal of volatile compounds did not improve lag-phase of FPA productivities at early stage,

rather than were left behind compared to non-detoxified slurry. Also, above it was shown that enzymes produced with non-detoxified slurry in enzyme assays produced 21% higher glucose yield, and 13% more xylose than enzymes produced with slurry containing partly detoxified filtrate. Therefore, it appears that volatile fraction of filtrate not only appear not to have inhibited enzyme production from *T. reesei*, but also improved the glucose yields of produced enzyme mixtures with pretreated rape straw filter cake substrate (see Figure 5). Further enrichment of medium in filtrate in partly detoxified Filtrate carbon source produced the significantly lowest FPA productivities (see Table 4 and Figure 2), and FPA yields (see Table 6 and Figure 3) of all carbon sources. Growth rate of *Trichoderma* was not analyzed because the experiments were done in 150 ml shake flasks with no oxygen control, and more than that contained insoluble carbon sources (data not shown), so that determination of produced biomass was complicated and unreliable [50]. An alternative source of information for cell growth was the consumption of soluble sugars present in carbon soure. For example, soluble sugars of filtrate (Detoxified Filtrate) were the highest for time zero (see Figure 4) among all carbon source as shown in Table 3, and decreased to levels similar to the other carbon sources after 8 days.

The observed low cell activity that was underscored by the low sugar consumption (data not shown) and the low FPA productivity (see Figure 2) could be attributed either to inhibition of cell metabolism by chemical inhibitors present in filtrate or to a deficient inducing system for enzyme production. Filtrate from WO pretreated rape straw contained soluble oligomers and monomers of cellulose and hemicellulose (see Table 3). The exact composition of soluble sugars was not analyzed, but an example of the distribution of soluble sugars filtrate can be seen from Qing et al. Sugar oligomers and monomers have shown to have inducing properties like for example monosaccharides (glucose, xylose, lactose), disaccharides (like cellobiose, sophorose, sorbose), and cello-oligomers, but their induction properties depend on compound [6,16,33,34]. On the other hand, it has been reported earlier [20,38,51] that filtrates from pretreated biomasses did not support growth and enzyme production in *Trichoderma* and *Aspergillus* strains. Comparing with other carbon sources of Table 3the partly detoxified filtrate (Detoxified Filtrate) contained the highest amount of volatile and non-

volatile inhibitors measured, except furfural content that was lower than undetoxified slurry. In brief, the source of inhibition of filtrate in all experiments containing filtrate was not clear, but it appears that is not caused by volatile compounds. Major non-volatile component detected was phenolics, but further elucidation on the causation of low FPA productivities of carbon sources containing filtrate (Slurry, Detoxified Slurry, and Detoxified Filtrate) is needed.

Detected βG activity was below 0.1 IU/ml in all tested carbon sources. Although *T. reesei* is a hypercellulase producer, expressed βGs are not secreted in the medium but are bound to the hyphen, therefore it is difficult to be recovered and measured [14,17,52]. Nevertheless, the pH of the fermentations were kept at pH close to 6, which has been indicated to favor expression of extracellular βG [53,54], without success in enzyme titters for secreted βG though. The obtained results for low βG are in accordance with Merino and Cherry (2007), who reported that cellulases produced from *T. reesei* need to be supplemented with βG enzyme equal to 5% of total protein [55]. This advice was followed when all enzyme mixtures (both commercial and produced) were tested on hydrolyzing pretreated rape straw substrate, and Novozym 188 was dosed for 100 IU/g dry matter of βG activity.

Economy of enzyme production must be very low to reach the market needs [56]. In enzyme production processes final product yields (translated from raw material costs), fermentation time (translated from electricity consumption) are the key parameters [56]. Filter cake was fermented in 7 days and slurry in 11 days, and FPA yields after these times were comparable. However, enzymes produced with slurry resulted to higher glucose and xylose yields from hydrolyzing pretreated rape straw filter cake. Therefore, the pretreated whole slurry was more effective enzyme mixture than the other fraction of pretreated rape straw.

For an economical point of view, it was suggested previously to use 5% of lignocellulosic biomass for production of enzymes, assuming total of the remaining 95% of the processed solid material saccharification to fermentable sugars for e.g. ethanol production [17,57]. Here FPA yield was 120 FPU/g filter cake spent. At the same time, 30 FPU/g filter cake were dosed

in enzyme hydrolysis assays. Doing the math, 20% of filter cake needs to be spent for enzyme production, so that enough enzymes will be produced to hydrolyze the 80% of filter cake. This result is far above the 5% indicated, and for this process design of enzyme production and enzyme saccharification of pretreated rape straw need improvements in biomass use efficiency, before can be implemented. Some ideas on increase of enzyme fermentation efficiency is to increase carbon source concentration that will increase enzyme titers also [58], but lower the FPA yields also. Results from literature have shown that use of fed-batch is the best mode for combine higher enzyme titers and improved FPA yields [58-60].

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Reference List

[1] Taherzadeh MJ, Karimi K. Enzyme-Based Hydrolysis Processes for Ethanol from Lignocellulosic Materials: A Review. Bioresources 2007; 2(4):707-38.

[2] Himmel ME. Biomass recalcitrance: engineering plants and enzymes for biofuels production (vol 315, pg 804, 2007). Science 2007; 316(5827):982.

[3] Bhat MK. Cellulases and related enzymes in biotechnology. Biotechnology Advances 2000; 18(5):355-83.

[4] Knauf M, Moniruzzaman M. Lignocellulosic biomass processing: A perspective. International Sugar Journal 2004; 106(1263):147-50.

[5] Kumar R, Wyman CE. Cellulase Adsorption and Relationship to Features of Corn Stover Solids Produced by Leading Pretreatments. Biotechnology and Bioengineering 2009; 103(2):252-67.

[6] Kubicek CP. The cellulase proteins of *Trichoderma reesei*: Structure, multiplicity, mode of action and regulation of formation. Advances in Biochemical Engineering/Biotechnology 1992; 45/1992.

[7] Olsen HS. Status and perspectives for second generation ethanol production. Getreidetwchnologie 7-10-2009;63(3):72-77. Germany. 10-3-2011.
Ref Type: Magazine Article

[8] Mohagheghi A, Grohmann K, Wyman CE. Production of Cellulase on Mixtures of Xylose and Cellulose. Applied Biochemistry and Biotechnology 1988; 17:263-77.

[9] Rumbold K, van Buijsen HJJ, Overkamp KM, van Groenestijn JW, Punt PJ, van der Werf MJ. Microbial production host selection for converting second-generation feedstocks into bioproducts. Microbial Cell Factories 2009; 8.

[10] Martinez D, Berka RM, Henrissat B, Saloheimo M, Arvas M, Baker SE et al. Genome sequencing and analysis of the biomass-degrading fungus Trichoderma reesei (syn. Hypocrea jecorina) (vol 26, pg 553, 2008). Nature Biotechnology 2008; 26(10):1193.

[11] Sukumaran RK, Singhania RR, Pandey A. Microbial cellulases - Production, applications and challenges. Journal of Scientific & Industrial Research 2005; 64(11):832-44.

[12] Suto M, Tomita F. Induction and catabolite repression mechanisms of cellulase in fungi. Journal of Bioscience and Bioengineering 2001; 92(4):305-11.

[13] Improving cellulose hydrolysis with new cellulase compositions. Chemical engineers annual meeting; 05; Cincinati, OH: American Institute of Chemical engineers; 2005.

[14] Kovacs K, Szakacs G, Zacchi G. Comparative enzymatic hydrolysis of pretreated spruce by supernatants, whole fermentation broths and washed mycelia of Trichoderma reesei and Trichoderma atroviride. Bioresource Technology 2009; 100(3):1350-7.

[15] Meyer AS, Rosgaard L, Sorensen HR. The minimal enzyme cocktail concept for biomass processing. Journal of Cereal Science 2009; 50(3):337-44.

[16] Olsson L, Christensen TMIE, Hansen KP, Palmqvist EA. Influence of the carbon source on production of cellulases, hemicellulases and pectinases by Trichoderma reesei Rut C-30. Enzyme and Microbial Technology 2003; 33(5):612-9.

[17] Bollok M, Reczey K. Cellulase enzyme production by various fungal strains on different carbon sources. Acta Alimentaria 2000; 29(2):155-68.

[18] Juhasz T, Szengyel Z, Reczey K, Siika-Aho M, Viikari L. Characterization of cellulases and hemicellulases produced by Trichoderma reesei on various carbon sources. Process Biochemistry 2005; 40(11):3519-25.

[19] Palmqvist E, HahnHagerdal B, Szengyel Z, Zacchi G, Reczey K. Simultaneous detoxification and enzyme production of hemicellulose hydrolysates obtained after steam pretreatment. Enzyme and Microbial Technology 1997; 20(4):286-93.

[20] Thygesen A, Thomsen AB, Schmidt AS, Jorgensen H, Ahring BK, Olsson L. Production of cellulose and hemicellulose-degrading enzymes by filamentous fungi cultivated on wet-oxidised wheat straw. Enzyme and Microbial Technology 2003; 32(5):606-15.

[21] Karakashev D, Thomsen AB, Angelidaki I. Anaerobic biotechnological approaches for production of liquid energy carriers from biomass. Biotechnology Letters 2007; 29(7):1005-12.

[22] Petersson A, Thomsen MH, Hauggaard-Nielsen H, Thomsen AB. Potential bioetanol and biogas production using lignocellulosic biomass from winter rye, oilseed rape and faba bean. Biomass & Bioenergy 2007; 31(11-12):812-9.

[23] Mandels M, Weber J. Production of Cellulases. Advances in Chemistry Series 1969;(95):391-&.

[24] Miller GL. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Analytical Chemistry 1959; 31(3):426-8.

[25] Berghem LER, Pettersson LG, Axiofredriksson UB. Mechanism of Enzymatic Cellulose Degradation - Purification and Some Properties of 2 Different 1,4-Beta-Glucan Glucanohydrolases from Trichoderma-Viride. European Journal of Biochemistry 1976; 61(2):621-30.

[26] Ghose TK. Measurement of Cellulase Activities. Pure and Applied Chemistry 1987; 59(2):257-68.

[27] Bjerre AB, Ploeger A, Simonsen T, Woidemann A, Schmidt AS. Quantification of solubilised hemicellulose from pretreated lignocellulose by acid hydrolysis and high performance liqui chromatography. 1996;Risoe-R-855. Risoe National Laboratory, Denmark.
Ref Type: Report

[28] Domingues FC, Queiroz JA, Cabral JMS, Fonseca LP. Production of cellulases in batch culture using a mutant strain of Trichoderma reesei growing on soluble carbon source. Biotechnology Letters 2001; 23(10):771-5.

[29] Rautio JJ, Bailey M, Kivioja T, Soderlund H, Penttila M, Saloheimo M. Physiological evaluation of the filamentous fungus Trichoderma reesei in production processes by marker gene expression analysis. Bmc Biotechnology 2007; 7.

[30] Smith MPT, Coward-Kelly G, Smith M, inventors. NOVOZYMES NORTH AMERICA INC, assignee. Producing cellulase in a host cell comprises cultivating host cell capable of producing cellulase, where pre-treated ligno-cellulosic material is added to induce cellulase production. patent WO2007005918-A2; EP1899476-A2; IN200706056-P4; US2008199908-A1; CN101223273-A; CA2613717-A1.

[31] Ilmen M, Saloheimo A, Onnela ML, Penttila ME. Regulation of cellulase gene expression in the filamentous fungus Trichoderma reesei. Applied and Environmental Microbiology 1997; 63(4):1298-306.

[32] Jorgensen H, Olsson L. Production of cellulases by Penicillium brasilianum IBT 20888 - Effect of substrate on hydrolytic performance. Enzyme and Microbial Technology 2006; 38(3-4):381-90.

[33] Mandels M, Reese ET. Induction of Cellulase in Trichoderma-Viride As Influenced by Carbon Sources and Metals. Journal of Bacteriology 1957; 73(2):269-78.

[34] Wang CH, Hseu TH, Huang CM. Induction of Cellulase by Cello-Oligosaccharides in Trichoderma-Koningii G-39. Journal of Biotechnology 1988; 9(1):47-59.

[35] Janas P, Targonsky Z, Mleko S. New inducers for cellulases production by Trichoderma reesei M-7. Electronic Journal of Polish Agricultural Universities 2002; 5(1):4.

[36] Yang B, Wyman CE. Effect of xylan and lignin removal by batch and flowthrough pretreatment on the enzymatic digestibility of corn stover cellulose. Biotechnology and Bioengineering 2004; 86(1):88-95.

[37] Klinke HB, Olsson L, Thomsen AB, Ahring BK. Potential inhibitors from wet oxidation of wheat straw and their effect on ethanol production of Saccharomyces cerevisiae: Wet oxidation and fermentation by yeast. Biotechnology and Bioengineering 2003; 81(6):738-47.

[38] Szengyel Z, Zacchi G, Reczey K. Cellulase production based on hemicellulose hydrolysate from steam-pretreated willow. Applied Biochemistry and Biotechnology 1997; 63-5:351-62.

[39] Durand H, Clanet M, Tiraby G. Genetic-Improvement of Trichoderma-Reesei for Large-Scale Cellulase Production. Enzyme and Microbial Technology 1988; 10(6):341-6.

[40] Schaffner DW, Toledo RT. Cellulase Production by Trichoderma-Reesei When Cultured on Xylose-Based Media Supplemented with Sorbose. Biotechnology and Bioengineering 1991; 37(1):12-6.

[41] Seidl V, Gamauf C, Druzhinina IS, Seiboth B, Hartl L, Kubicek CP. The Hypocrea jecorina (Trichoderma reesei) hypercellulolytic mutant RUT C30 lacks a 85 kb (29 gene-encoding) region of the wild-type genome. Bmc Genomics 2008; 9.

[42] Eveleigh DE, Cuskey S, Montenecourt B. Production of Hypercellulolytic Mutants of Trichoderma. Abstracts of Papers of the American Chemical Society 1978; 175(MAR):56.

[43] Klinke HB, Ahring BK, Schmidt AS, Thomsen AB. Characterization of degradation products from alkaline wet oxidation of wheat straw. Bioresource Technology 2002; 82(1):15-26.

[44] Cantarella M, Cantarella L, Gallifuoco A, Spera A, Alfani F. Effect of inhibitors released during steam-explosion treatment of poplar wood on subsequent enzymatic hydrolysis and SSF. Biotechnology Progress 2004; 20(1):200-6.

[45] Delgenes JP, Moletta R, Navarro JM. Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by Saccharomyces cerevisiae, Zymomonas mobilis, Pichia stipitis, and Candida shehatae. Enzyme and Microbial Technology 1996; 19(3):220-5.

[46] Klinke HB, Thomsen AB, Ahring BK. Potential inhibitors from wet oxidation of wheat straw and their effect on growth and ethanol production by Thermoanaerobacter mathranii. Applied Microbiology and Biotechnology 2001; 57(5-6):631-8.

[47] Palmqvist E, Hahn-Hagerdal B. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresource Technology 2000; 74(1):25-33.

[48] Zaldivar J, Ingram LO. Effect of organic acids on the growth and fermentation of ethanologenic Escherichia coli LY01. Biotechnology and Bioengineering 1999; 66(4):203-10.

[49] Zaldivar J, Martinez A, Ingram LO. Effect of selected aldehydes on the growth and fermentation of ethanologenic Escherichia coli. Biotechnology and Bioengineering 1999; 65(1):24-33.

[50] Rapp P, Grote E, Wagner F. Formation and Location of 1,4-Beta-Glucanases and 1,4-Beta-Glucosidases from Penicillium-Janthinellum. Applied and Environmental Microbiology 1981; 41(4):857-66.

[51] Schmidt AS, Bailey MJ, Vaari A, Thomsen AB, Tenkanen M. Production of fungal plant cell wall degrading enzymes on wet-oxidised wheat straw xylan. ESPOO: TECHNICAL RESEARCH CENTRE FINLAND; 2000.

[52] Andric P, Meyer AS, Jensen PA, Dam-Johansen K. Effect and Modeling of Glucose Inhibition and In Situ Glucose Removal During Enzymatic Hydrolysis of Pretreated Wheat Straw. Applied Biochemistry and Biotechnology 2010; 160(1).

[53] Juhasz T, Szengyel Z, Szijarto N, Reczey K. Effect of pH on cellulase production of Trichoderma reesei RUT C30. Applied Biochemistry and Biotechnology 2004; 113:201-11.

[54] Juhasz T, Egyhazi A, Reczey K. beta-glucosidase production by Trichoderma reesei. Applied Biochemistry and Biotechnology 2005; 121:243-54.

[55] Merino ST, Cherry J. Progress and challenges in enzyme development for Biomass utilization. BERLIN: SPRINGER-VERLAG BERLIN; 2007.

[56] Tufvesson P, Lima-Ramos J, Nordblad M, Woodley JM. Guidelines and Cost Analysis for Catalyst Production in Biocatalytic Processes. Organic Process Research & Development 2011; 15(1):266-74.

[57] Galbe M, Larsson M, Stenberg K, Tengborg C, Zacchi G. Ethanol from wood: Design and operation of a process development unit for technoeconomic process evaluation. WASHINGTON: AMER CHEMICAL SOC; 1997.

[58] Persson I, Tjerneld F, HahnHagerdal B. Fungal Cellulolytic Enzyme-Production - A Review. Process Biochemistry 1991; 26(2):65-74.

[59] Ahamed A, Vermette P. Enhanced enzyme production from mixed cultures of Trichoderma reesei RUT-C30 and Aspergillus niger LMA grown as fed batch in a stirred tank bioreactor. Biochemical Engineering Journal 2008; 42(1):41-6.

[60] Lee SM, Koo YM. Pilot-scale production of cellulase using Trichoderma reesei rut C-30 in fed-batch mode. Journal of Microbiology and Biotechnology 2001; 11(2):229-33.

Paper III

Arvaniti E, A Thygesen, Z Kádár, AB Bjerre, JE Schmidt Assessing conditions of simultaneous saccharification and fermentation for ethanol production from pre-treated rape straw, Biomass and Bioenergy, 2010 submitted

III ASSESSING CONDITIONS OF SIMULTANEOUS SACCHARIFICATION AND FERMENTATION FOR ETHANOL PRODUCTION FROM PRE-TREATED RAPE STRAW

Contributors: Efthalia Arvaniti, Anders Thygesen, Zsófia Kádár, Anne Belinda Thomsen

Abstract

Ethanol production using wet-oxidised (WO) rape straw whole slurry was optimised during Simultaneous Saccharification and Fermentation (SSF) experiments by testing three pure cultures of *S. cerevisiae* and a baker's yeast. Parameters in focus were thermotollerance, low pH, and dry matter (DM). Evaluation was based on achieved ethanol yields. SSF of whole slurry with baker's yeast showed that adjustment of pH before inoculation was important for achieving high ethanol yields (68%). There were no significant difference in the ethanol yields at 32 °C (70-75%) and 37 °C (72-76%) for the three pure cultures. However, SuMo (isolated from baker yeast) and Turbo (isolated from a brewing yeast) achieved higher ethanol yields at 40 °C (64-66%), compared to ATCC 96581 (57%) after 115 hours of SSF. Increasing DM from 12.5% to 16% DM during SSF experiments with SuMo yeast at 37 °C for 120 hours did not have any significantly influence on the ethanol yields (75-76%). While further increase the DM content to 18% resulted in a decrease of the ethanol yield to 68%. In SSF experiments in a fermenter of 1 litre working volume with a DM of 12.5% DM and a fixed pH to 4.8 resulted in ethanol yield (63%) compared to shake flask reference. Moreover, lactic acid was formed as by-product (11 g/l).

1 Introduction

The conventional method for producing bioethanol from lignocellulosic biomass is facilitated by thermo-chemical pretreatment step of biomass that opens the structure and reduces crystallinity, then enzyme saccharification of carbohydrates followed by fermentation of released sugars to ethanol by microorganism [1,2]. After fermentation the ethanol-rich broth is distilled up to 95%, and then ethanol is dehydrated to reach standard fuel requirements [3].

According to Wingren et al. [4] the four industrial benchmarks for low cost bioethanol (spanning from thermo-chemical pretreatment of biomass, to enzyme saccharification, ethanol fermentation, and ethanol distillation) are: 1) Process water economy; 2) inhibitor tolerance; 3) ethanol yield; and 4) specific ethanol productivity [5]. **Process water economy** translates to high dry matter content of biomass in pretreatment and SSF, which implies low fresh water consumption, and more concentrated streams [6]. Concentrated streams significantly reduce overall capital and operational costs of ethanol production by use of smaller volume setup for same production output [7] or by increasing energy efficiency in evaporation and distillation [4]. On the other hand, higher osmolarity stresses both the performances of enzymes [8-10] and yeasts [11-13], resulting in lower product yields and productivities. **Inhibitor tolerance** of yeast to compounds produced during pretreatment [14,15] and enzyme saccharification like low (Molecular Weight) MW organic acids, furans, phenolics [16], is essential when concentrated streams are fermented. **Ethanol yield** close to theoretical maximum (90-95%) [17] is critical for low cost bioethanol production. It has been estimated that the single highest cost in ethanol production is feedstock, accounting for one third of the production cost [4,18-20]. For this, use of C6 and C5 sugars needs to be utilized for ethanol production [5]. Finally, volumetric **ethanol productivity** [21] (gram ethanol l^{-1} h^{-1}) is translated to processing time of SSF, and decreases by inhibition [15,22]. A solution to overcome inhibition is to increase the cell density of yeast in fermentation, but cell cultivation has also a production cost, as a function of the added nutrients. Specific productivity (gram ethanol g

biomass^{-1} h^{-1}) on the other hand, gives information on the fermenting efficiency of the yeast, and allows benchmarking of yeasts [5].

Finding a robust, osmotolerant, and inhibitor-tolerant fermenting strain is important for obtaining a viable ethanol production in industrial scale [23,24]. *Saccharomyces cerevisiae* is by far the most popular ethanol fermenting strain tested for decades at industrial scale. The prevalence of these microorganisms is the high ethanol yield (80-90%), the high volumetric ethanol productivity (1-3 gram ethanol l^{-1} h^{-1}), its growing and fermenting capacity at various oxygen environments, and the high ethanol (80-100 g/l) and inhibitor tolerance [25,26].

Pretreated rape straw is an agricultural residue that has been proposed for ethanol production [27]. Previous research has shown that after pretreatment by wet oxidation at 205 ^{o}C for 3 min with 12 bar of oxygen gas pressure and featured with presoaking in water, not only the washed filter cake but the whole slurry was fermented (Unpublished data). This allowed avoid washing and increase water economy [11,27,28]. In this study, three different monocultures of *S. cerevisiae* strains and a dry baker's yeast formulation are tested in SSF experiments. The DNA of the three monocultures was compared to identify differences. Substrate for SSF was pre-treated rape straw by wet oxidation (WO). In SSF experiment, yeasts were tested at stress conditions: high temperature up to 40 ^{o}C, low pH (below 4), and increased osmolarity (high dry matter SSF) in SSF experiments. The best SSF conditions and yeast strain are assessed by the highest ethanol yields.

2 Materials and methods

2.1 Rape straw

Oilseed rape straw (*Brassica Napus*, variery Carakas) was collected from fields of Hornsherred near Lyngby, Denmark in August 2007, air-dried to 90-95% humidity and stored at room temperature. The straw

was milled in 2 mm particle size by knife mill and pretreated first by soaking in water for 20 minutes at 80 °C in 6% dry matter concentration (DM), and then by wet oxidation (WO) technique in a 2 liter loop reactor with 12 bar of oxygen gas at 205 °C for 3 minutes. These conditions have been found optimal for ethanol production (unpublished data in our laboratory). After pretreatment, the solids were separated from the liquids by vacuum filtration with a 0.1 mm mesh nylon filter and both filter cake (14.9±1.1% DM) and filtrate were stored at 4 °C. In some experiments, that higher DM solids were needed, filter cake was additionally pressed through cotton cloth to 26% DM. The DM of the fibers was measured gravitationally by Mettler Toledo HR-83P. Upon use the filter cake and filtrate were mixed again.

2.2 Yeasts

Three monocultures of *Saccharomyces cerevisiae* strains and one dry baker's yeast formulation were used: 1) "SuMo" yeast was isolate from a commercial dry baker's yeast in our laboratorium; 2) *Saccharomyces cerevisiae* ATCC 96581; 3) "Turbo" yeast isolate from commercial dry brewer's yeast (Alcotec 24 hour pure Turbo Super yeast, Hambleton Bard Ltd, UK); 4) dry baker's yeast packaging Malteserkors Gær, Denmark.

The monocultures were stored at -80 °C, and inoculated under sterile conditions in 80 ml sterilized YPD medium, containing 20 g/l glucose, 20 g/l peptone, and 10 g/l bacto yeast extract. The pre-cultures were shaken at 30 °C for 24 hours, and subsequently transferred to 1L sterile growth medium for another 24 hours. Then, yeast cells were centrifuged, and resuspended in water. Their DM content was determined by drying at 105 °C. Dry baker's yeast was not subjected to pre-culture.

DNA analysis of the yeast cell from the three monocultures was as follows: The cells were first cultivated overnight in YPD medium (same as before) at 30 °C. Cells were then harvested by centrifugation at 2000 rpm for 5 min, and the rDNA was isolated from the cells as described previously [29]. The extracted rDNA was subsequently

cleaved with the restriction enzyme EcoRI by following the manufactures recommendation (Fermentas, #0274). Finally, fragments were isolated by agarose gel electrophoresis at 400 V/m for 60 min, and photographed in UV lamp.

2.3 Simultaneous Saccharification and Fermentation

Experiments under different conditions and with different yeasts were performed. The list of SSF experiments is listed in Table 1. All SSF experiments were preceded by a 24-hours liquefaction step according to experimental setup of Varga et al. [10]. All experiments were run in duplicates (both shake flasks and fermenter).

Experiments testing SSF temperatures, yeast cells, and dry matter contents of biomass were done follows: Shake flasks of 250 ml are loaded with 10 gram of filter cake of pre-treated rape straw and adjusted to a certain DM by diluting with filtrate that are adjusted first at pH 4.8 with 6 M NaOH. Slurry was mixed with 15 FPU/g DM Celluclast 1.5L, (Novozymes, Denmark) and Novozym 188 (Novozymes, Denmark) in 5:1 volumetric ratio. The flasks were shaken at 50 ^{o}C for 24 hours at 120 rpm. After the 24 hour liquefaction step, liquefied slurry was cooled-down to the appropriate SSF temperature (see Table 1) pH was adjusted again to 4.8 with 6 M NaOH, and then 20 FPU/g DM Celluclast 1.5L and Novozym 188 at 5:1 volumetric ratio, 0.8 g/l urea and 2.5 g/l *S. cerevisiae* yeast cells were added. The flasks were flashed with nitrogen, topped with yeast lockers, and incubated on rotary shaker at 120rpm for 120 hours. The time zero of SSF starts with addition of yeast. The experiment ended after 115-120 hours.

In SSF experiments studying the pH (experiments PH/L and PH/H see Table 1), the experimental setup was similar as above except for the following: only for PH/H the pH was re-adjusted to 4.8 before adding the yeast. In PH/L experiment the pH was not re-adjusted before inoculating the yeast. Also, dry baker's yeast was used instead of pure culture, and the temperature of SSF (after 24 hour liquefaction) was only at 32 ^{o}C.

Table 1 Liquefaction and SSF experiments of this study are clustered in three groups. The parameter that was variable in each cluster is highlighted in bold. In the first cluster the pH of SSF is studied, in the second cluster the SSF temperature and the fermentation strain, and in the third cluster the DM content of liquefaction/SSF. The pH of SSF at time zero refers to time before inoculating the yeast.

	Name code	Host strain	SSF Temperature (°C)	pH of SSF at time zero (-)	DM content (%)
pH	PH/L	Baker's yeast	32	**3.9**	12.5
	PH/H	Baker's yeast	32	4.8	12.5
Thermotollerance of yeasts	SM32	**SuMo**	**32**	4.8	12.5
	SM37	SuMo	**37**	4.8	12.5
	SM40	SuMo	**40**	4.8	12.5
	AT32	**ATCC96581**	**32**	4.8	12.5
	AT37	ATCC96581	**37**	4.8	12.5
	AT40	ATCC96581	**40**	4.8	12.5
	TB32	**TurBo**	**32**	4.8	12.5
	TB37	TurBo	**37**	4.8	12.5
	TB40	TurBo	**40**	4.8	12.5
Increased dry matter	HI1	SuMo	37	4.8	**16.0**
	SM-H2	SuMo	37	4.8	**17.9**

Finally, in the scaled up SSF to 900 ml first liquefaction was done in a plastic cylinder shake flask of 1L and then the liquefied slurry was transfer to a 2 litter anaerobic fermenter. The enzymes, nutrients, and clean culture of yeast were added as described above. The pH was adjusted automatically to 4.8 by H_2SO_4 1M or NaOH 6M. Mixing in the fermenter was done with propeller at 40 rpm. Samples of the slurry were taken every 1-3 days and stored at 4 °C until analysis. The experiment ended after 120 hours.

2.4 Analysis

The composition of filter cake and filtrate is shown in Table 2. Filter cake was first dried at 105 °C and macerated with a knife-mill to 1 mm, and then analyzed for total sugars (glucose, xylose, and arabinose, and

mannose) content after strong acid hydrolysis and Klason lignin as described earlier [27].

Filtrate was analyzed for total sugars (glucose, xylose, and arabinose, and mannose) through weak acid hydrolysis as described earlier [27], free sugars, low molecular weight organic acids (formic acid, acetic acid, lactic acid, succinic acid, glycolic acid), furans (2-furfural, 5-hydroxymethyl-furfural, and 2-furoic acid), glycerol, and ethanol by HPLC. A Shimadzu Corp HPLC (Kyoto, Japan) system equipped with BioRad HPX-87H column (Amminex) at 63 ^{o}C, was used with 4 mM H_2SO_4 as eluent at 0.6 ml/min flow rate. The detector for furans was a Diode array SPD-M10AVP (Shimadzu Corp, Kyoto, Japan) and for the other compounds was a RID-10A RI-detector (Shimadzu Corp, Kyoto, Japan).

Table 2 Composition of filter cake (gram per 100 gram wet insoluble solids, WIS), and filtrate (grams per liter) of rape straw, after pre-treated by soaking in water at 6% DM at 80 ^{o}C for 20 minutes, and Wet-oxidized with 12 bar O_2 gas at 205 ^{o}C for 3 minutes. The polymer and oligomer sugars are calculated as monomer equivalents (hydrated form). NCWM: Non cell-wall material; water soluble substances, pectins proteins, extractives, etc.

Component filter-cake	Concentration (g/100g WIS)	Component of filtrate	Concentration (g/l)
Glucan	58,4	Glucan oligomers	0,9
Xylan	14,0	Xylan oligomers	6,4
Arabinan	0.6	Arabinan oligomers	0,3
Lignin	23.1	Glucose	0,2
Ashes	2.6	Xylose	0,4
NCWM	13.4	Arabinose	0,2
		Acetic acid	0.9
		Formic acid	0.9
		Furfural	0.1
		Total phenolics	1.3
		pH	3.9

The liquid samples from fermenter in scaled up experiments were analyzed also for phenolic monomers (15 different compounds) as well as total dissolved phenolic compounds. The total phenolic content was measured spectrophotometrically by the Prussian blue method [30]. The monomeric phenolic compounds were selectively extracted from the samples by solid phase extraction on polystyrene diphenylbenzene polymer columns Isolute ENV+ 100mg 1ml SPE by Biotage as described by Klinke et al. (2002) [14], and analyzed by Gas Chromatography (GC). GC was a HP 6890 series system equipped with flame ionization detection and a Zebron 2B-5MSi column (Phenomenex), and Helium gas as eluent 0.7 ml/min.

2.5 Calculations

Ethanol yield % of SSF was:

$$Y_{EtOH}\% = \frac{Ethanol\ concentration\ after\ SSF}{Ethanol\ potential\ of\ cellulose\ added\ in\ SSF} \times 100\% \qquad (1)$$

In case residual glucose was detected after SSF, it was translated into **residual glucose yields**. This was calculated in the same fashion as (eq.1), but numerator was different:

$$Y_{Glc}\% = \frac{Residual\ glucose\ concentration\ after\ SSF}{Glucose\ potential\ of\ cellulose\ added\ in\ SSF} \times 100\% \qquad (2)$$

Total glucose yields (projected) were calculated by summing the residual glucose yield and normalized ethanol yield into glucose yield equivalents:

$$Y^{T}_{Glc}\% = \frac{Residual\ glucose\ conc.after\ SSF + \frac{Ethanol\ conc.after\ SSF}{0.51}}{Glucose\ potential\ of\ cellulose\ added\ in\ SSF} \times 100\% \qquad (3)$$

Where 0.51 is the stoichiometric variable of glucose to ethanol conversion.

Progress of ethanol fermentation is calculated from equivalents of CO_2 vent during fermentation or SSF (in shake flask experiments), or by sampling (only in fermenter experiments). For the first case, ethanol equivalent for each gram of CO_2 produced is 1,045 assuming that only

ethanol is produced. From this amount of ethanol titers (g/l) and ethanol yields % are calculated.

Ethanol productivity is measured as volumetric ethanol productivity (gram ethanol l^{-1} h^{-1}) with data either

$$Q_{EtOH} = \frac{\Delta C_{EtOH}}{\Delta t} \qquad \text{(eq.2)}$$

Where ΔC_{EtOH} is the ethanol concentration difference (g/l) between to sampling times and t is the time difference (hours) between to two sampling times.

Standard deviation was used for analyzing the dispersion of duplicate experiments, and stems all graphic illustrations in figures. The standard deviation was produced using STDEVA formula of Excel software for an array of data that cumulatively normalized in a single average value.

One-way analysis of variance analyzed data for 5% or 10% significance level by of grouped experiments (treatments) with different conditions (levels). For example, ethanol yields at SSF at 32^{o}C were analyzed together. In the case, the group analyzed with ANOVA contained more than two levels, and results were found significantly different by ANOVA, **Newman and Keuls statistical analysis** tool was applied at 5% or 10% significance level (in accordance to ANOVA), to identify in which levels of the treatment were significantly different. Newman and Keuls was calculated with DSAASTAT macro v. 1.101 (Perugia, Italy) in Excel software.

3 Results

3.1 Effect of pH on the fermentation

The aim in these experiments was to study the effect of pH on the SSF using baker's yeast. For this, the SSF was done under two different pH (see Table 1). Experiment PH/H was re-adjusted to pH 4.8 before inoculating the yeast (after 24 hours liquefaction), whereas PH/L was not re-adjusted before inoculating the yeast, and in this case pH was 3.9±0.1. The ethanol yields and ethanol productivities, calculated from CO_2 release during SSF, are presented in Figure 1.

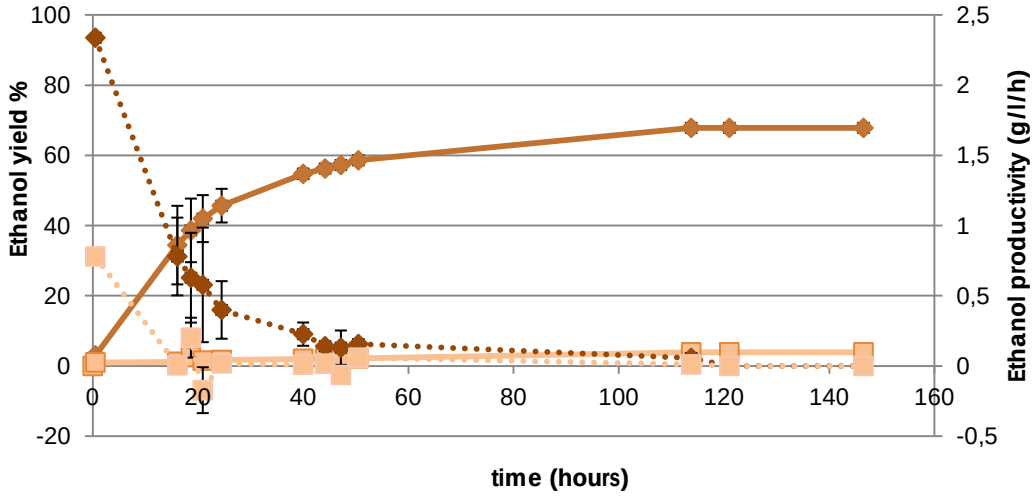

Figure 1 Ethanol yields % and ethanol productivities of SSF experiments at different start pH. Orange lines shows SSF experiment at start pH 4.8 (PH/H experiment), and pink line the ethanol yields at start pH 3.9 (not adjusted after 24 hour liquefaction step, PH/L experiment). Straight lines are ethanol yields, and dotted lines ethanol productivities. The experiments ran in duplicates, and error bars show standard deviation of the duplicates. Time zero starts when yeast is added.

In Figure 1, when pH was re-adjusted before inoculating the yeast (experiment PH/H) ethanol productivity was 2.3 g l^{-1} h^{-1}, and after 114 hours of SSF the ethanol yield was 68%. The experiment was extended to 146 hours without improvement in ethanol yields. After SSF, no residual glucose or lactic acid was detected for PH/H (data not shown). In the case that pH was not readjusted before inoculating the yeast (experiment PH/L) ethanol productivity was 0.8 g g l^{-1} h^{-1}, and ethanol yield was close to 5%. Further time extension of the experiment PH/L to 930 hours increased ethanol yield to 26% (data not shown).

Therefore, re-adjusting pH to 4.8 facilitated ethanol production in SSF of whole slurry with baker's yeast (S. cerevisiae).

The acetic acid content of filtrate in shake flasks before inoculating the enzymes (time zero of liquefaction) was 0.9 g/l (see Table 2), and in the end of fermentation in SSF was 2.6 g/l for both experiments (data not shown).

3.2 Selection of temperature and yeast strain

The DNAs of these *S. cerevisiae* strains (called SM, AT, and TB) was first analysed for genotypic differences. The rDNA of the three strains was digested by a mixture of restriction enzymes and the DNA fragments are shown in Figure 2. The fragments were distributed between 1700 and 4500 base pairs in 8 bands (see reference ladder on the left side of Figure 2). The strains have five common bands shown with black lines at Figure 2. In addition to these, fragments of Turbo (TB) and ATCC96581 (AT) have at least one distinct band that is shown by arrows. This is indication that these three strains have different DNA sequences, and thereby they are not the same strains.

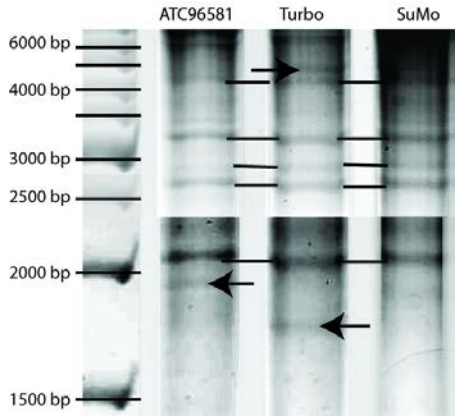

Figure 2. Electrophoresis gel of rDNA fragments from three *S. cerevisiae* strains (ATCC96581, Turbo, and SuMo yeast), after being digested with ECO RI enzymes. On the left side lies the reference ladder with indicating sizes of base pairs of DNA chains. The common bands of the DNA of the strains are indicated with lines, while the distinct bands are shown with arrows.

In the following SSF experiments the most thermotollerant strain among the three *S. cerevisiae will be identified*. SSF with whole pre-treated slurry is used at 12.5% DM, and the pH after liquefaction was re-adjusted to 4.8 in all SSF experiments. The final obtained ethanol yields are compared.

In Figure 3 the obtained residual glucose yields and ethanol yields after SSF are shown. The ethanol and glucose yields can be compared after considering that stoichiometry equals 1% glucose yield with 0.5% ethanol yield. The ethanol yields obtained from the three tested yeasts were not significant different at SSF temperature 32 °C ranging from 69-74%, or at 37°C ranging 70-74%. Only for SSF experiments at 40 °C the ethanol yields were found significantly. The lowest yields was observed for ATCC96581 (55%) while the yields for the other two strains (SuMo, and Turbo) that ranged from 62-65%.

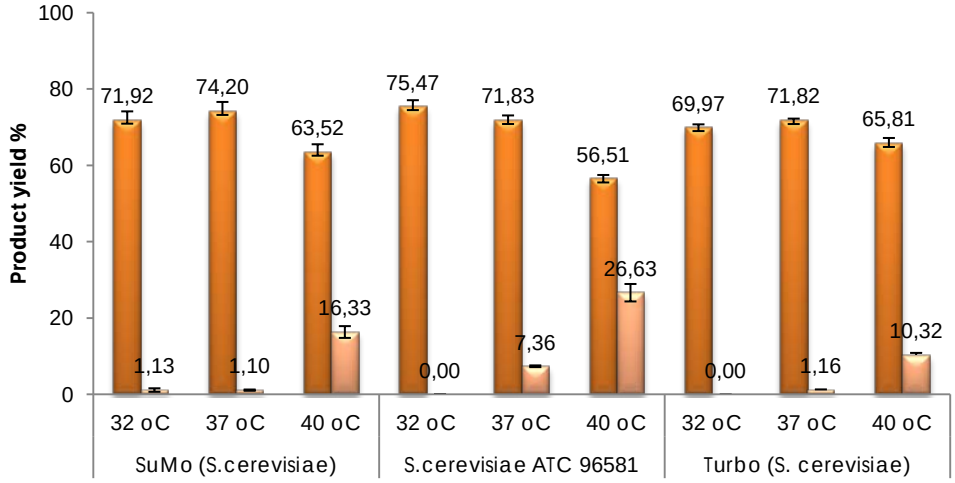

Figure 3 Product yields of SSF experiments with three S. cerevisiae yeasts SuMo (SM), ATC96581 (AT), and Turbo (TB) at three SSF temperatures 32, 37, and 40 oC. Orange bars are the obtained ethanol yields (%), and the pink bars are the residual glucose yields. The error bars show the standard deviation of the duplicates.

By comparing ethanol yields of yeasts obtained for the same temperature, ethanol yields for SuMo at 40 °C SSF and for ATCC96581 at 40 °C SSF were found significantly lower at 55% and 61% respectively, compared to their respective ethanol yields at lower temperatures that were standing 72-76% for both strains. From Figure 3it is shown that the fermentation at 40 °C was not so effective as at lower temperatures.

Ethanol yields of SuMo (SM) and ATCC96581 (AT) were significantly lower compared to lower SSF temperatures. In case of Turbo yeast SSF at 37 °C (72%) was significantly higher than at 40 °C (66%) (see Figure 3). The results are summarized in Table 3. Thereby, all three yeasts have achieved comparable ethanol yields at both 32°C and 37 °C and lower at 40.

Table 3 Results from ANOVA and Newman-Keuls analysis at 5% confidence of the ethanol yields achieved for each host strain. According to ANOVA, all ethanol yields were significantly different for each host. After Newman-Keuls analysis, ethanol yields are ranked from a tobz, with a the highest and b the lowest significant value.

Host strain	SSF temperature		
	32 °C	37 °C	40 °C
SuMo	a	a	b
ATCC96581	a	a	b
Turbo	ab	a	b

From Figure 3, information on the residual glucose yields are retrieved. At 32°C, all yeasts left no residual glucose ATCC96581. At 37°C glucose yields of SuMo and Turbo were significantly lower at 37 °C 0-1% than ATCC96581 that was 7% (see Figure 3). Finally, at 40 °C the significantly highest residual glucose yield was 27% for ATCC96581(see Figure 3) .

Finally, in all combinations of tested SSF temperatures and yeast, a not significantly different amount of lactic acid of 0.3 g/l was detected.

Recapitalizing, the most thermotollerant strains were SuMo and Turbo given that exhibited the highest ethanol yields at 37 and 40 °C and lowest non-fermented ethanol yield.

3.3 Effect of dry matter content in SSF

In this set of experiments, the effect of increasing the concentration of solids (DM) of whole slurry in SSF experiments was investigated. The SSF experiment SM37 was selected from previous paragraph as base experiment with 12.5% DM, and experiment HI1 with 16% DM, and HI2 with 17.9% DM were compared (see Table 1).

Results from both ethanol yields and residual glucose yields are presented in Figure 4. There was no significant difference in the ethanol yields of the three experiments at 5% significance. At 10% significance, ethanol yields were significantly lowest for Hi2 (68%) compared to ethanol yields for the lower DM ranging 75-76%. The ethanol concentration after 115 hours of SSF was significantly lowest for SM37 with 30 g/l, compared to higher DM that ranged 37-38 g/l ethanol (data not shown). Moreover, residual glucose yields of Hi2 (18% DM) were significantly higher (13%) than the other two SSF experiments with lower DM content ranging 1-4% (see Figure 4).

The lactic acid content was in the range of 0.2-0.3 g/l for all experiments.

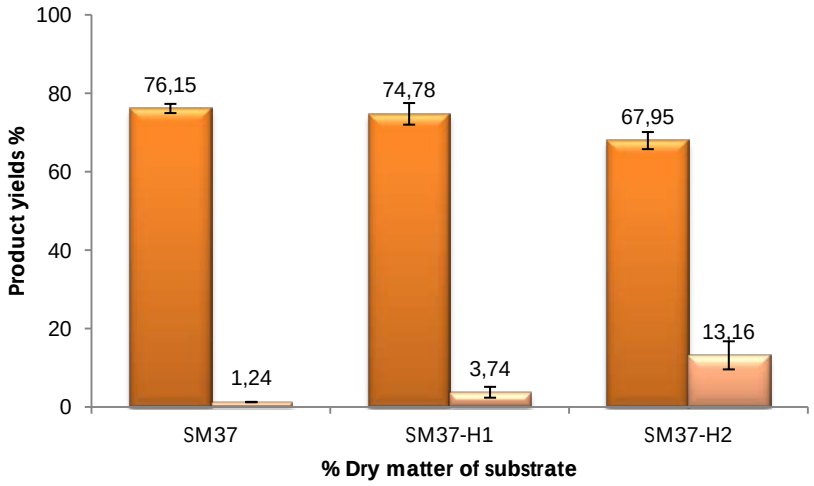

Figure 4 Product yields of SSF experiments after 116 hours at three different dry matter contents SM37 (12.5% DM), SM37-H1 (16% DM), and SM37-H2 (17.9% DM), according to Table 11. Orange bars show the obtained ethanol yields and pink bars the residual glucose yields. The error bars show standard deviations of the duplicates.

3.4 SSF in fermenter with constant pH

SSF experiment SM37 (see Table 1) was scaled up 11-fold in a fermenter and pH was fixed to 4.8±0.1 with pH control. The ethanol yields in the fermenter after liquefaction for 24 hours and SSF for 120 hours was 63%, which was significantly lower (5% significance) than 73% ethanol yield

achieved in shake flasks for experiment SM37 in shake flask as described earlier (see also Figure 3).

The main compounds found in SSF experiments in the fermenter are presented in Table At time zero where yeast was inoculated of SSF 22 g/l glucose (see Figure 5) corresponding to 53% glucose yield (data not shown) were released by enzymes. And after 20 hours of SSF glucose concentration was 4 g/l. However, after 120 hours glucose concentration in one fermenter was 9 g/l (now called #1) and in the other fermenter (duplicate) there was no glucose detected (now called #2). Ethanol significantly increased to 19 g/l after 20 hours of SSF with ethanol productivity 0.93 g $l^{-1}h^{-1}$, after 44 hours stabilised to 25-26 g/l of ethanol were produced. Beyond 44 hours ethanol production was not improved.

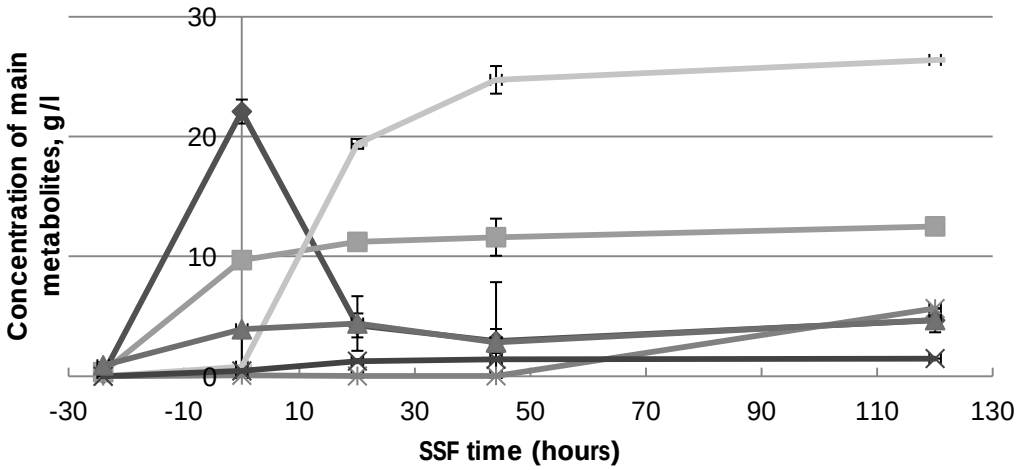

Figure 5 Concentrations of majopr components present in SSF present in the fermenter. Liquefaction starts at -24 hours, and time zero of SSF is when yeast is inoculated: Glucose (x symbol), Ethanol (-), Xylose (square), lactic acid (star), Glycerol (rhombe), acetic acid (triangle). The error bars show the standard deviation of the duplicates.

After 120 hours fermenter #2 contained 11.2 g/l lactic acid (see Table 4), and Fermenter #1 contained 9 g/l glucose instead. Glycerol significantly increased in the first 44 hours (1.4-1.5 g/l, see Figure 5). Concentration of acetic acid in filtrate at time zero of liquefaction was

0.9 g/l (see Table 2). In SSF, acetic acid concentration did not significantly changed ranging from 2.8-4.7 g/l (see Figure 5).

Table 4 summarises all the minor metabolites and other compounds detected in SSF medium. Concentration of formic acic did not significantly changed in 120 hours. At time zero of SSF furfural was 0.074 g/l, furoic acid was 0.015 g/l, and HMF was 0.013 g/l. During SSF furfural halved, HMF was depleted, and furoic acid increased to 0.038 g/l. From phenolic compounds, ferulic acid, vanillic acid and coumaric acid decreased in the system in 120 hours, and other compounds like vanillyl alcohol increased.

Table 4 List of minor components present in SSF experiments in the fermenter; including cellobiose, fermentation metabolites, and inhibitors from sugar and lignin degradation. Analysis ran for 3 to 4 sampling times. After ± is shown the standard deviation of the duplicates. NA: Not analysed.

Minor components	Concentration (mg/L)			
	0 hours	20 hours	44 hours	120 hours
Cellobiose	0	0	55±78	95±64
Formic acid	1113±NA	1130±24	1098±148	1118±23
Furfural	74±3	45±0	40±0	38±0
5-HMF	13±0	3±0	0	0
2-furoic acid	15±0	16±0	16±1	38±1
Guaiacol	16±1	16±2	NA	10±8
Syringol	4±0	2±0	NA	3±3
4-hydroxybenzylalcohol	1±0	3±0	NA	3±1
Vanillyl alcohol	10±1	12±1	NA	25±20
Vanillin	1±0	5±0	NA	3±2
Syringaldehyde	3±2	7±1	NA	4±4
4-hydroxybenzaldehyde	2±0	7±2	NA	3±1
3,4,5-Trimethoxyacetophenon	3±1	59±3	NA	29±29
4-hydroxybenzoic acid	6±3	5±5	NA	4±2
Ferulic acid	19±5	15±18	NA	3±2
Vanillic acid	43±1	20±0	NA	17±5
Syringic acid	0	0	NA	9±12
Coumaric acid	19±1	12±13	NA	6±4

4 Discussion

By re-adjusting pH from 3.9 to 4.8 before inoculating dry baker's yeast in SSF experiments ethanol productivity increased 3-fold, and ethanol yield reached 68% after 120 hours.

It has been reported that *S. cerevisiae* grows and ferments ethanol even at pH below 3 but presence of acetic acid (and more likely other organic acids [22]) in combination to low pH is detrimental to yeast metabolism [31,32]. Total acetic acid content of filtrate before liquefaction was 0.9 g/l that is not encountered as inhibitory from literature [33]. After SSF with re-adjusted pH, the acetic acid content was 2.6 g/l. Such a concentration combined with a pH 3.5-4.5 was found to inhibit ethanol productivity by 75%, according to Pampulha et al [34]. Low pH undissociates organic acids (like acetic acid [22] and 4-hydroxybenzoic acid [35]) that assists passive diffusion [14] through the cell wall causing proton stress and finally cell lysis [36].

Due to the 3-fold increase of ethanol productivity by adjustment of pH to 4.8 before inoculation of baker's yeast, adjustment was applied in other SSF experiments with pure cultures. However, in the latter case the experiment was not repeated with the pure cultures, therefore the pH effect was not proved for the pure cultures.

The three yeasts (SuMo, Turbo, and ATCC96581) had different sources, and DNA analysis indicated that the three yeasts had more likely different genotype [37]. When these strains were tested for their thermotollerance in SSF experiments at 32 and 37 ^{o}C for 115 hours, they did not achieve significantly different ethanol yields, ranging from 72-76%. At 40 ^{o}C SuMo (a baker yeast isolate [38]) and Turbo (a brewing yeast isolate) were the most thermotollerant (64-66% ethanol yield), compared to ATCC 96581 (57% ethanol yield). ATCC96581 at 40 ^{o}C exhibited the significantly lowest ethanol yield and the significantly highest residual glucose yield, which makes it the least thermotollerant strain of all tested. The low ethanol yield together with high residual glucose yields of ATCC96581 might be described by inhibition of both ethanol production and glucose uptake rate [39].

In SSF experiments at 40 °C yeasts were more stressed by temperature, and less by other factors like organic acids, pH, or ethanol accumulation, but combination of many stresses makes difficult to evaluated source of inhibition. ATCC96581 was isolated from a spent sulphite liquor fermentation plant in Sweden [40], and is known as an inhibitor-tolerant/acidotolerant strain that can ferment ethanol in undetoxified acid pre-treated wood filtrates at pH 4.5 at 30 °C [22,40]. Geng et al. [41] recommended ATCC96581 as a candidate for production of bioethanol in SSF due to its relatively high temperature, acid, and ethanol tolerance. Nevertheless, the low ethanol yields achieved here at 40 °C compared to the other two strains might be explained either by low thermotollerance, or loss of acid tolerance as a result of synergism with other inhibitors. Pampulha et al. [34] reported that tolerance of yeast to acetic acid decreases when ethanol is present as a result of synergism. From the data, the source of inhibition of ATCC96581 though cannot be identified.

By increasing temperature from 32 °C to 40 °C in SSF 6-8% more total glucose yield was released by enzymes in 115 hours. Given that enzyme saccharification is 1-2 orders of magnitude lower than typical fermentation rate (typical 1-3 gram ethanol l^{-1} h^{-1} [42], indicates the necessity of finding a thermotollerant or thermophilic strain for SSF. Increase of temperature beyond 40 °C has been reported to halt growth and fermenting capacity of the yeast [43,44], and adaptation is cannot go beyond 3-5 °C [45]. Instead, other biotechnology tools are required to improve heat tolerance of ethanol fermenting strains [45]. Given the prevalence of the yeast in SSF, research is directed to improve thermal properties of yeast either by mutagenesis or molecular biology techniques [46,47]. More than that attempts focus in facilitating C6 and C5 sugar fermentation with yeast, and thereby improve ethanol yields per gram of biomass [5].

From all the obtained results, it appears that SuMo yeast (baker's yeast) and Turbo yeast (brewer yeast) could be the best candidates for temperature adaptation to higher temperatures in SSF [45], starting with 37°C as optimal SSF temperature in the given setup.

Increase of DM in SSF experiments with SuMo yeast at 37 °C, up to 16% did not create any significant difference in ethanol yields (75-76%) and residual glucose yields (1-4%). However, beyond that at 17.9% DM, ethanol fermentation was inhibited so that ethanol yields were lower (68%) and glucose accumulated (13%). As a result, the maximum ethanol concentration did not exceed 38 g/l.

In an attempt to increase the final ethanol concentration before distillation, DM of SSF was increased from 12.5 to 16 and 17.9%. In literature, increase of DM during SSF has been reported to decrease ethanol yields [20,48], which offsets the benefits of working at high DM.

During SSF with high DM content the osmolarity of the wort increases during enzyme saccharification (organic acids, sugars, salts), that challenged both enzymes activities and yeast growth and fermentation. To avoid this, one solution is to wash the fibres before SSF. For example, Mohagheghi et al. [49] reported a linear ethanol yield decrease as DM of increased from 7.5 to 20%, but these results followed washing of pre-treated wheat straw before SSF. Also, when Varga et al. (2004) raised the DM during SSF with washed pre-treated corn stover found maximum ethanol yield (77%) at 17% DM, but beyond that DM dropped sharply to 4% ethanol yield. In hereby experiments, pre-treated filter cake were not washed, and whole slurry was used instead at elevated DM, thereby water economy in SSF was controlled down.

From the total glucose yields, there is no evidence that enzymes were inhibited by increasing the DM of SSF. However, the amount of enzymes used was relatively high (35 FPU/g DM), thereby inhibition can be masked easier. At lower enzyme loadings though, the inhibition of enzymes is more apparent at high DM [10,11].

According to Wingren et al [4], economy of ethanol distillation requires a threshold ethanol concentration, which is drawn down to 5% w/v ethanol. Below that value, distillation costs are increasing exponentially, and ethanol distillation is not recommended [6]. For achieving the minimum 5% w/v ethanol concentration in hereby SSF experiments, it was calculated that with a hypothetical 90% ethanol yield, 18.5% DM of pre-treated rape straw was needed. However, the ethanol yield at

17.9% DM was only 38 g/l (3.8%) and this is because ethanol yield was only 68%. However, maximum ethanol yield is important to limit ethanol production costs [4]. From the available data, it cannot be concluded the reason of yeast inhibition at elevated DM in SSF. It is speculated though that is a combination of inhibition caused by low pH and high organic acids content released during high DM enzyme hydrolysis of pre-treated rape straw.

A fermenter was used for scaling up 11-fold the SSF experiment with SuMo yeast at 37 ^{o}C and 12.5% DM of pretreated slurry. The fermenter also allowed keeping constant pH at 4.8. Achieved ethanol yields in fermenter were lower (63%) than in shake flask, in which pH was not control after inoculation. Also, significant increase of residual glucose yields was observed in one fermenter. In the other fermenter, glucose was depleted but lactic acid was produced instead. Lactic acid was only a minor side-product in most experiments tested in this study (0-0.3g/l), but SSF experiments in the fermenter with fixed pH allowed production of 11 g/l of lactic acid in one fermenter (out of two parallels).

Contamination by lactic acid bacteria (LAB) was not testified in microscope, but contamination by LAB is common phenomenon in ethanol production setups (from sugar, starch, or lignocellulose) [25]. To battle contamination by LAB different counter-measurements are taken in industry like low pH (below 5) ethanol fermentation [50], increase density of yeast [51], or use of natural bactericides (like hop [25]). Production of lactic acid was observed after 44 hours of SSF. Belated proliferation of LAB after 55 hours of SSF with pre-treated softwood has been reported also from Stenberg et al. [52]. The reason behind this might be that yeast metabolizes inhibitors (thereby detoxifying) present in the wort [52], and also provide nutrients essential for LAB [53].

Glucose accumulated (9 g/l) in the fermenter that was not contaminated, and gas production stopped already at 110 hours (although SSF lasted for 120 hours). Thereby ethanol fermentation halted. However, the reason behind incomplete fermentation in SSF cannot be explained. Glycerol that is formed mainly during biosynthesis

can be used as a cell growth marker [54]. Glycerol significantly increased in the first 44 hours (1.4-1.5 g/l), but later on stabilized and moreover, it did not differentiate between the two fermenters. This hold evidence that yeast growth halted after 44 hours.

One of the potential inhibitors that could explain yeast growth inhibition was (total) acetic acid that ranged from 3-5 g/l after the end of SSF. But, because pH is kept 4.8 such a concentration is not believed to inhibit yeast, Oura [33] reported that 5 g/l acetic acid (undissociated form) inhibited yeast growth [33]. Since, pK_a of acetic acid is 4.75, the acetic acid content was half of the reported.

Furfural is the strongest inhibitor of yeast [55] (LD_{50} for *E. Coli* was 2.5 g/l [56]). Microbial reduction of furfural produces furfuryl alcohol that is less inhibitory [57] (LD_{50} at 4 g/l [57]). Furfural (75 mg/l maximum) was well below the reported inhibitory levels (furfural at 0.5 g/l has been reported to reduce growth by 47% and ethanol production by 43% [55]. Other furans like hydroxyl-methyl-furfural (5-HMF) was 13 mg/l, which was well below the inhibitory level (1 g/l 5-HMF 65% reduced cell growth and 71% of ethanol production [55]). Therefore, inhibition to yeast caused by identified furans was very weak in this experiment.

Also phenolic aldehydes were identified in SSF medium (see In Table 7). It is well known that phenolic compounds are inhibitors acting synergistically with other degradation products [22,56], and that can act as electro acceptors for yeast; hence phenolic acids acids like ferulic acid, vanillic acid and coumaric acid decreased in the system in 120 hours, whereas more reduced compounds like vanillyl alcohol increased.

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Reference List

[1] Lange JP. Lignocellulose conversion: an introduction to chemistry, process and economics. Biofuels Bioproducts & Biorefining-Biofpr 2007; 1(1):39-48.

[2] Margeot A, Hahn-Hagerdal B, Edlund M, Slade R, Monot F. New improvements for lignocellulosic ethanol. Current Opinion in Biotechnology 2009; 20(3):372-80.

[3] Kumar S, Singh N, Prasad R. Anhydrous ethanol: A renewable source of energy. Renewable & Sustainable Energy Reviews 2010; 14(7):1830-44.

[4] Wingren A, Galbe M, Zacchi G. Techno-economic evaluation of producing ethanol from softwood: Comparison of SSF and SHF and identification of bottlenecks. Biotechnology Progress 2003; 19(4):1109-17.

[5] Hahn-Hagerdal B, Karhumaa K, Fonseca C, Spencer-Martins I, Gorwa-Grauslund MF. Towards industrial pentose-fermenting yeast strains. Applied Microbiology and Biotechnology 2007; 74(5):937-53.

[6] Zacchi G, Axelsson A. Economic-Evaluation of Preconcentration in Production of Ethanol from Dilute Sugar Solutions. Biotechnology and Bioengineering 1989; 34(2):223-33.

[7] Gnansounou E, Dauriat A. Techno-economic analysis of lignocellulosic ethanol: A review. Bioresource Technology 2010; 101(13):4980-91.

[8] Kristensen JB, Felby C, Jorgensen H. Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. Biotechnology for Biofuels 2009; 2.

[9] Hodge DB, Karim MN, Schell DJ, McMillan JD. Soluble and insoluble solids contributions to high-solids enzymatic hydrolysis of lignocellulose. Bioresource Technology 2008; 99(18):8940-8.

[10] Varga E, Klinke HB, Reczey K, Thomsen AB. High solid simultaneous saccharification and fermentation of wet oxidized corn stover to ethanol. Biotechnology and Bioengineering 2004; 88(5):567-74.

[11] Georgieva TI, Hou XR, Hilstrom T, Ahring BK. Enzymatic hydrolysis and ethanol fermentation of high dry matter wet-exploded wheat straw at low enzyme loading. Applied Biochemistry and Biotechnology 2008; 148(1-3):35-44.

[12] Mohagheghi A, Tucker M, Grohmann K, Wyman C. High Solids Simultaneous Saccharification and Fermentation of Pretreated Wheat Straw to Ethanol. Applied Biochemistry and Biotechnology 1992; 33(2):67-81.

[13] Zhao H, Jones CIL, Baker GA, Xia S, Olubajo O, Person VN. Regenerating cellulose from ionic liquids for an accelerated enzymatic hydrolysis. Journal of Biotechnology 2009; 139(1):47-54.

[14] Klinke HB, Ahring BK, Schmidt AS, Thomsen AB. Characterization of degradation products from alkaline wet oxidation of wheat straw. Bioresource Technology 2002; 82(1):15-26.

[15] Klinke HB, Olsson L, Thomsen AB, Ahring BK. Potential inhibitors from wet oxidation of wheat straw and their effect on ethanol production of Saccharomyces cerevisiae: Wet oxidation and fermentation by yeast. Biotechnology and Bioengineering 2003; 81(6):738-47.

[16] Klinke HB, Thomsen AB, Ahring BK. Potential inhibitors from wet oxidation of wheat straw and their effect on growth and ethanol production by Thermoanaerobacter mathranii. Applied Microbiology and Biotechnology 2001; 57(5-6):631-8.

[17] Rumbold K, van Buijsen HJJ, Overkamp KM, van Groenestijn JW, Punt PJ, van der Werf MJ. Microbial production host selection for converting second-generation feedstocks into bioproducts. Microbial Cell Factories 2009; 8.

[18] Galbe M, Zacchi G. Pretreatment of lignocellulosic materials for efficient bioethanol production. Biofuels 2007; 108:41-65.

[19] Klein-Marcuschamer D, Oleskowicz-Popiel P, Simmons BA, Blanch HW. Technoeconomic analysis of biofuels: A wiki-based platform for lignocellulosic biorefineries. Bionass and Bioenergy. In press 2010.

[20] Sassner P, Galbe M, Zacchi G. Techno-economic evaluation of bioethanol production from three different lignocellulosic materials. Biomass & Bioenergy 2008; 32(5):422-30.

[21] Alfani F, Gallifuoco A, Saporosi A, Spera A, Cantarella M. Comparison of SHF and SSF processes for the bioconversion of steam-exploded wheat straw. Journal of Industrial Microbiology & Biotechnology 2000; 25(4):184-92.

[22] Palmqvist E, Grage H, Meinander NQ, Hahn-Hagerdal B. Main and interaction effects of acetic acid, furfural, and p-hydroxybenzoic acid on growth and ethanol productivity of yeasts. Biotechnology and Bioengineering 1999; 63(1):46-55.

[23] Banerjee S, Mudliar S, Sen R, Giri B, Satpute D, Chakrabarti T et al. Commercializing lignocellulosic bioethanol: technology bottlenecks and possible remedies. Biofuels Bioproducts & Biorefining-Biofpr 2010; 4(1):77-93.

[24] Vintila T, Vintila D, Neo S, Tulcan C, Hadaruga N. Simultaneous hydrolysis and fermentation of lignocellulose versus separated hydrolysis and fermentation for ethanol production. Romanian Biotechnological Letters 2011; 16(1):106-12.

[25] Sanchez OJ, Cardona CA. Trends in biotechnological production of fuel ethanol from different feedstocks. Bioresource Technology 2008; 99(13):5270-95.

[26] Claassen PAM, de Vrije T. Non-thermal production of pure hydrogen from biomass: HYVOLUTION. International Journal of Hydrogen Energy 2006; 31(11):1416-23.

[27] Petersson A, Thomsen MH, Hauggaard-Nielsen H, Thomsen AB. Potential bioetanol and biogas production using lignocellulosic biomass from winter rye, oilseed rape and faba bean. Biomass & Bioenergy 2007; 31(11-12):812-9.

[28] Felby C, Klinke HB, Olsen HS, Thomsen AB. Ethanol from wheat straw cellulose by wet oxidation pretreatment and simultaneous saccharification and fermentation. 2003.

[29] Pedersen MB. Dna-Sequence Polymorphisms in the Genus Saccharomyces .1. Comparison of the His4 and Ribosomal-Rna Genes in Lager Strains, Ale Strains and Various Species. Carlsberg Research Communications 1983; 48(5):485-503.

[30] Graham HD. Stabilization of the Prussian Blue Color in the Determination of Polyphenols. Journal of Agricultural and Food Chemistry 1992; 40(5):801-5.

[31] Narendranath NV, Power R. Relationship between pH and medium dissolved solids in terms of growth and metabolism of lactobacilli and Saccharomyces cerevisiae during ethanol production. Applied and Environmental Microbiology 2005; 71(5):2239-43.

[32] Taherzadeh MJ, Niklasson C, Liden G. Acetic acid - friend or foe in anaerobic batch conversion of glucose to ethanol by Saccharomyces cerevisiae? Chemical Engineering Science 1997; 52(15):2653-9.

[33] Oura E. Reaction-Products of Yeast Fermentations. Process Biochemistry 1977; 12(3):19-&.

[34] Pampulha ME, Loureiro V. Interaction of the Effects of Acetic-Acid and Ethanol on Inhibition of Fermentation in Saccharomyces-Cerevisiae. Biotechnology Letters 1989; 11(4):269-74.

[35] Verduyn C, Postma E, Scheffers WA, Vandijken JP. Effect of Benzoic-Acid on Metabolic Fluxes in Yeasts - A Continuous-Culture Study on the Regulation of Respiration and Alcoholic Fermentation. Yeast 1992; 8(7):501-17.

[36] Verduyn C, Postma E, Scheffers WA, Vandijken JP. Energetics of Saccharomyces-Cerevisiae in Anaerobic Glucose-Limited Chemostat Cultures. Journal of General Microbiology 1990; 136:405-12.

[37] Lopes MD, Soden A, Martens AL, Henschke PA, Langridge P. Differentiation and species identification of yeasts using PCR. International Journal of Systematic Bacteriology 1998; 48:279-86.

[38] Jensen M, Thomsen ST. 2nd Generation Bioethanol from Lucerne - Sustainable Solution for Future Bioenergy [Roskilde: Technical University of Denmark; 2009.

[39] Mensonides FIC, Schuurmans JM, de Mattos MJT, Hellingwerf KJ, Brul S. The metabolic response of Saccharomyces cerevisiae to continuous heat stress. Molecular Biology Reports 2002; 29(1-2):103-6.

[40] Linden T, Peetre J, HahnHagerdal B. Isolation and Characterization of Acetic Acid-Tolerant Galactose-Fermenting Strains of Saccharomyces-Cerevisiae from A Spent Sulfite Liquor Fermentation Plant. Applied and Environmental Microbiology 1992; 58(5):1661-9.

[41] Geng AL, Wang ZK, Lai KS, Tan MWY. Bioenergy II: Comparison of Laboratory and Industrial Saccharomyces cerevisiae Strains for their Stress Tolerance. International Journal of Chemical Reactor Engineering 2010; 8.

[42] Claassen PAM, van Lier JB, Contreras AML, van Niel EWJ, Sijtsma L, Stams AJM et al. Utilisation of biomass for the supply of energy carriers. Applied Microbiology and Biotechnology 1999; 52(6):741-55.

[43] Kadar Z, Szengyel Z, Reczey K. Simultaneous saccharification and fermentation (SSF) of industrial wastes for the production of ethanol. Industrial Crops and Products 2004; 20(1):103-10.

[44] Edgardo A, Carolina P, Manuel R, Juanita F, Jaime B. Selection of thermotolerant yeast strains Saccharomyces cerevisiae for bioethanol production. Enzyme and Microbial Technology 2008; 43(2):120-3.

[45] Banat IM, Nigam P, Singh D, Marchant R, Mchale AP. Ethanol production at elevated temperatures and alcohol concentrations: Part I - Yeasts in general. World Journal of Microbiology & Biotechnology 1998; 14(6):809-21.

[46] Davidson JF, Whyte B, Bissinger PH, Schiestl RH. Oxidative stress is involved in heat-induced cell death in Saccharomyces cerevisiae. Proceedings of the National Academy of Sciences of the United States of America 1996; 93(10):5116-21.

[47] Ye YR, Zhu Y, Pan L, Li LL, Wang XI, Lin Y. Gaining insight into the response logic of Saccharomyces cerevisiae to heat shock by combining expression profiles with metabolic pathways. Biochemical and Biophysical Research Communications 2009; 385(3):357-62.

[48] Ohgren K, Rudolf A, Galbe M, Zacchi G. Fuel ethanol production from steam-pretreated corn stover using SSF at higher dry matter content. Biomass & Bioenergy 2006; 30(10):863-9.

[49] Mohagheghi A, Tucker M, Grohmann K, Wyman CE. High Solid Simultaneous Saccharification and Fermentation of Pretreated Wheat Straw to Ethanol. Abstracts of Papers of the American Chemical Society 1989; 198:35-MBTD.

[50] Kadar Z, Maltha SF, Szengyel Z, Reczey K, De Laat W. Ethanol fermentation of various pretreated and hydrolyzed substrates at low initial pH. Applied Biochemistry and Biotechnology 2007; 137:847-58.

[51] Thomas KC, Hynes SH, Ingledew WI. Effect of lactobacilli on yeast growth, viability and batch and semi-continuous alcoholic fermentation of corn mash. Journal of Applied Microbiology 2001; 90(5):819-28.

[52] Stenberg K, Galbe M, Zacchi G. The influence of lactic acid formation on the simultaneous saccharification and fermentation (SSF) of softwood to ethanol. Enzyme and Microbial Technology 2000; 26(1):71-9.

[53] Challinor SW, Rose AH. Interrelationships Between A Yeast and A Bacterium When Growing Together in Defined Medium. Nature 1954; 174(4436):877-8.

[54] Verduyn C, Postma E, Scheffers WA, Vandijken JP. Physiology of Saccharomyces-Cerevisiae in Anaerobic Glucose-Limited Chemostat Cultures. Journal of General Microbiology 1990; 136:395-403.

[55] Delgenes JP, Moletta R, Navarro JM. Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by Saccharomyces cerevisiae, Zymomonas mobilis, Pichia stipitis, and Candida shehatae. Enzyme and Microbial Technology 1996; 19(3):220-5.

[56] Zaldivar J, Martinez A, Ingram LO. Effect of selected aldehydes on the growth and fermentation of ethanologenic Escherichia coli. Biotechnology and Bioengineering 1999; 65(1):24-33.

[57] Zaldivar J, Martinez A, Ingram LO. Effect of alcohol compounds found in hemicellulose hydrolysate on the growth and fermentation of ethanologenic Escherichia coli. Biotechnology and Bioengineering 2000; 68(5):524-30.

Curriculum Vitae

Efthalia Arvaniti was born in 26th of October 1978, in Athens, Greece. She grew up in downtown in Kipseli, and graduated 30th general high school of Athens. She was very keen to study Chemistry and she gave national-level exams to enter the University of Patras, department of Chemistry in 1998. During her study, she focused in Nuclear chemistry, and Environmental Chemistry. She was very inspired by a bachelor course of Renewable Sources of Energy and Chemical Storage, and decided to study Environmental Sciences. In 2004, she moved in the Netherlands to Wageningen University, to follow a Master of Science in Environmental Sciences. She specialised in Environmental technologies and use of industrial and agricultural residues for production of Biofuels. She did her master thesis in the sub-department of Environmental Technology of Wageningen University, and her master internship in Food and Biobased research group of Wageningen UR. After graduation, she was invited in Risø National Laboratory for Sustainable Energy of Technical University of Denmark for pursuing a PhD degree. During her PhD study, she visited the Department of Applied Biotechnology and Food Science of Budapest University of Technology and Economics for completing part of her experimental work.

Risø DTU is the National Laboratory for sustainable Energy. Our research focuses on development of energy technologies and systems with minimal effect on climate, and contributes to innovation, education, and policy. Risø has large experimental facilities and interdisciplinary research environments, and includes the national centre for nuclear technologies.

Risø DTU
National Laboratory for Sustainable Energy
Danish Technical University

Frederiksborgvej 399
Postboks 49
4000 Roskilde
Phone +45 4677 4677
Fax +45 4677 5688

www.risoe.dtu.dk