Processing of lignocelluloses using physical and biotechnological methods

Extraction of hemicelluloses, enzyme fermentation and hydrolysis

Ph. D. THESIS

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2008

1 Introduction

Biomass containing cellulose, hemicelluloses and lignin is called lignocellulose. The total amount of biomass in Hungary is about 350-360 million tons. The renewable part represents 105-110 million tons per year, which means a huge raw material potential. Beside building- and furniture industries lignocelluloses were used so far only by the textile, pulp and paper industries. Nowadays biorefinery concept comes to the front. During biorefinery the whole raw material is converted to value added products and not only parts of it are utilized.
In my PhD work I dealt with one possible biorefinery concept. I studied wide spectra of lignocellulosic substrates for the production of high molecular mass hemicellulose extractives and bioethanol. The former can be used as value added component of biodegradable polymers and hydrogels, the latter could partly replace fossil fuels.

2 Aims of the work

The aim of my PhD studies was better understanding of the utilization of lignocelluloses for bioethanol purposes. In order to achieve this goal, I had the following objectives in my research.

1. To study if microwave assisted heat extraction is a suitable tool for the separation of hemicellulose fraction from corn fiber. To determine maximum achievable molecular weight and the necessary treatment conditions.
2. To prove or confute by lab scale fermentor experiments the positive effect of tris-maleic acid buffer on the cellulase enzyme activities previously obtained in our group in shake flask cultivations with *Trichoderma reesei* RUT C30.
3. To test a genetically modified mutant of *Trichoderma reesei* (provided by ROAL Oy, Finland) in lab scale fermentor. The novel strain contained the CBHI gene of a thermoactive microorganism. The goal was to study the stability, fermentability of the mutant while comparing to the parental Rut-C30 strain.
4. To study the effect of cellulase enzymes on the hydrolysis of several lignocelluloses with and without the use of "helper" enzymes.
5. Comparison of home made enzymes with commercial Celluclast 1.5L cellulase product in the hydrolysis of lignocellulosic substrates.

3 Literature background

To study bioconversion of lignocelluloses knowledge on their structural features is essential. As their name suggests, they consist of three main components, cellulose, hemicelluloses and lignin.
Cellulose is a homopolysaccharide, with the smallest repeating unit of cellobiose, which is the dimmer of glucose units linked by β-1,4-linkages. Cellulose is a linear polymer which can build inter and intramolecular H-bonds. The macromolecule has crystalline and amorphous regions as well, is insoluble in water, chemically stable and resists enzymes to a great extent.
Hemicelluloses have shorter chains than cellulose, but have various substituents, thus are less crystalline, and more reactive. They consist of D-xylose, L-arabinose, D-galactose, D-glucose, D-glucuronic acid, D-mannose, and rarely L-fucose, L-rhamnose and L-galactose. They can be grouped as xylans, xyloglucans, mannans, mixed linkage β-glucans and arabionogalactans.
Aromatic polymers containing phenyl-propane subunits are termed as ***lignins***. Their three main monomers are called as *p*-coumar-alcohol, conyferil-alcohol and synapil-alcohol.

Components of lignocelluloses form a rather complex three dimensional structure, which is resistant to chemicals and microorganisms. Therefore, it is necessary to pretreat these materials in order to relax the structure. Physicochemical methods are applied in most cases. In industrial scale steam pretreatment proved to be the most effective. It's lab scale screening method is the microwave assisted heat treatment. This was used for the treatment of corn fiber to loosen up the lignocellulose structure, meanwhile extracting high molecular weight hemicellulose.

Other valuable products obtained from the hydrolysis of lignocelluloses are monosaccharides, which can further be converted to ethanol. Hydrolysis is performed by cellulases and hemicellulases. Three main groups of cellulases can be distinguished: endoglucanases, cellobiohydrolases, and β-glucosidases. Endoglucanases attack the cellulose chain randomly, while cellobiohydrolases cleave cellobiose from the end of the chains. β–glucosidases complete the process with releasing glucose from the cellobiose units. These enzymes can be produced by fermentation; but fungi produce them only in the presence of inducing agents.

Since cellulose fibers are highly covered by hemicelluloses in the lignocellulose structure, the accessibly surface area available for cellulases is rather small. This can be changed by pretreatment of the raw material, but in spite of this, there are still remaining hemicellulose moieties on the fibers. With the application of so called "helper" enzymes beside cellulases in the hydrolysis of lignocellulosic substrates this problem can be solved. Hemicellulases degrade hemicellulose polymer to smaller oligomers, or even monomers, thus cellulases can attack cellulose better.
These helper enzymes are hemicellulases (xylanases, xyloglucanases, ferulic acid esterases, acetil xylan esterases, galactosidases, arabinofuranosidases, fucosidases, mannanases), but also lignin degrading mangane peroxidases, laccases, lignin peroxidases, and pectin degrading endopolygalacturonidases, exopolygalacturonidases and pectin methyl esterases.

4 Materials and Methods

4.1 Microwave assisted heat extraction of corn fiber hemicellulose

Destarched corn fiber was impregnated with water for 6 hours before microwave irradiation. Occasionally acid or alkaline catalyst was also used. The impregnated raw material was treated for 5 minutes at the temperature of 100, 130, 160, 180, 200 or 210°C in Milestone MLS-1200 equipment. Afterwards, the fiber fraction was separated from the hemicellulose containing liquid fraction by vacuum filtration.

The monosaccharide composition of the extracted hemicellulose was determined using high performance liquid chromatography (HPLC–PAD, Dionex, USA), while the molecular weight was determined using size exclusion chromatography (SEC). The SEC system consisted of Superdex 75 and 200 columns connected in an FPLC system (GE Healthcare, Sweden). Detection was performed measuring refractometric index (RI detector, Japan) and UV detection (GE Healthcare) at the wavelength of 280 nm.

4.2 Enzyme fermentation

Delignified spruce pulp (Solka Floc 200), lactose-1-hydrate and steam pretreated corn stover were used as carbon sources for the fermentation of *Trichoderma reesei* Rut-C30 and RF6026 strains on two different media in shake flasks and lab scale fermentor (31 liter Biostat CDCU-3). To follow the cultivation supernatants were analyzed for protein and reducing sugar content, and several enzyme activities (filter paper activity, ß-glucosidase, xyloglucanase, xylanase, CBHI and EGI activities).

Substrate of filter paper activity measurements was Whatman No.1. filter paper, ß-glucosidase activity was determined by *p*-nitrophenyl-β-D-glucopyranosid, xyloglucanase by *Tamarind* xyloglucan and xylanase by oat spelt xylan. CBHI and EGI activities were determined using 4-methylumbelliferil-β-D-lactoside as substrate. The products of enzymatic reactions were measured using spectrophotometer.

4.3 Enzymatic hydrolysis

In the hydrolysis experiments hydrolysis of several differently pretreated lignocelluloses (barley straw, wheat straw, corn stover, willow, spruce, reed canary grass) was studied using purified cellulase (CBH I and II, EG II, β-glucosidase), and hemicellulase (xylanase and xyloglucanase) enzymes.

Hydrolytic potential of home made *T. reesei* enzymes fermented on different carbon sources (Solka Floc, lactose, steam pretreated corn stover) was studied also in hydrolysis experiments.

Enzymatic hydrolysis was performed in lab scale, in a volume of 5.0 or 2.5 ml, 0,05M Na-acetate buffer at pH 4,8-5,0 and 45-50°C, agitated by magnetic stirring (500 rpm) in test tubes. Concentration of the substrate was 10 g/l carbohydrate or 10 g/l glucan.

Samples were boiled for 10 minutes in order to stop the enzymatic reaction, than centrifuged and the supernatants analyzed for reducing sugar concentration and monosaccharide composition using HPLC (Dionex DX500 chromatographic system, Dionex Corp., USA).

5 Results

My doctoral work covers three big areas; hemicellulose extraction from corn fiber using microwave assisted heat treatment, and two biotechnological fields, enzyme production and enzymatic hydrolysis. In this chapter the main results are summarized.

Hemicellulose fraction was obtained from corn fiber using microwave assisted heat treatment. Molecular weight distribution and monosaccharide composition was analyzed. To retrieve high molecular weight hemicellulose fraction is not easy, since as a result of the treatment not only the lignocellulose structure is loosening up, but also the hemicelluloses degrade to smaller or longer oligomers and monomers.
Effect of treatment temperature on molecular weight and hemicellulose (xylan) yield was examined without application of any catalyst. Temperature ranged from 100 to 210°C. At lower temperatures no hemicellulose could be extracted, probably since it was too low for the disruption of lignocellulosic structure. At 180°C the yield was 20%, while molecular weight of the hemicellulose fraction was 1.42 x 10^5. Increasing the temperature caused increase in the yield, but decrease in the molecular weight (Figure 1). At the highest treatment temperature (210°C) only 6 x 10^4 molecular weight could be achieved. This is probably due to the phenomena often seen in case of acetyl-group containing hemicelluloses: at elevated treatment temperatures the acetyl-groups split off the polymer, forming acetic acid, which catalyzes the degradation of glycosil linkages. (pH measurements support this hypothesis.)

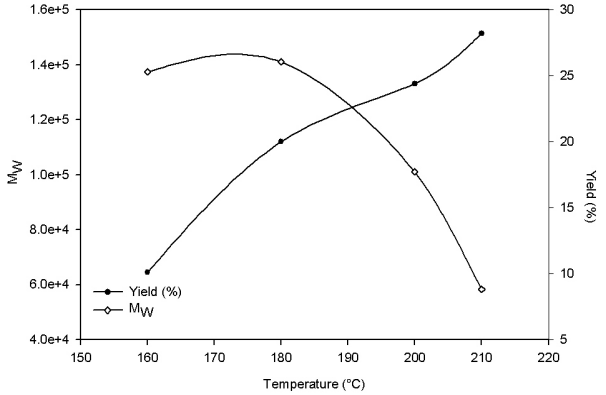

Figure 1: Effect of treatment temperature on the yield (●) and molecular weight (□) of microwave extracted hemicellulose

Effect of different catalysts was studied at 180°C for 5 minutes. Samples were impregnated with 0,025 or 0,5% sulphuric-acid or sodium hydroxide solution prior microwave treatment. Both the molecular weight and the yield decreased due to acid catalysis. Using alkaline catalyst higher molecular weight could be reached (172 000 and 136 000), which is probably due to the neutralizing effect of alkaline. Unfortunately the yield was rather law in these cases.

In lab scale fermentor experiments *Trichoderma reesei* Rut-C30 was compared to a genetically modified mutant strain (*T. reesei* RF6026). The modified strain contained the cbh1/cel7A gene of *Thermoascus aurantiacus* under the strong *T. reesei* cbh1/cel7A promoter. (In the RF6026 strain the native *T. reesei* cbh1/cel7A gene was deleted.) During fermentation production of the target protein (CBH I/Cel7A) was successful, and proved by enzyme activity measurements carried out at 50 and 70°C (Figure 2). CBH I enzyme

produced by Rut-C30 strain lost 70-80% of its activity at 70°C, while RF6026 strain carrying the *Thermoascus cbh1* performed well on elevated temperature as well.

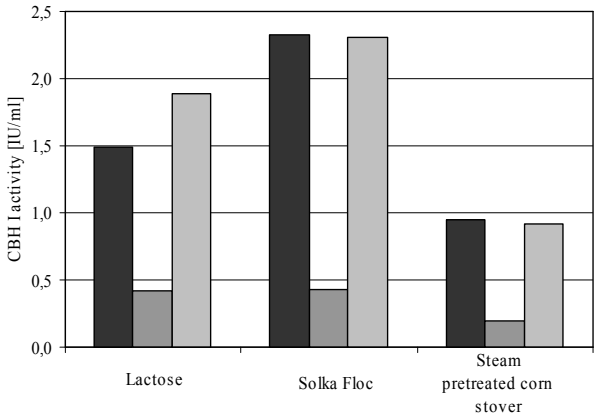

Figure 2: Enzyme activities of *T. reesei* Rut-C30 CBH I at 50°C (■) and 70°C (■) and RF6026 at 70°C-on (■), enzymes were produced on various carbon sources.

Shake flask experiments carried out previously in our research group proved that 0.05M tris-maleic acid buffer keeps the pH constant at the desired 5.6-5.8 range, and what is more the level of β-glucosidase production increases. With lab scale fermentor experiments I wanted to judge the mechanism of this positive effect, whether it is due to the constant pH only, or to the buffer components themselves. In fermentor – equipped with automatic pH control – it was proved that not (only) the stable pH, but also the buffer components are needed to obtain this positive effect.

The role of hemicellulases (xylanase, xyloglucanase) applied together with cellulases was studied in hydrolysis experiments using purified enzyme components. In the cell wall of higher plants cellulose, hemicelluloses and lignin construct a complex network structure. The hemicelluloses cover the cellulose fibers, thus access of cellulase enzymes to their substrate is limited. This can be enhanced by application of hemicellulase enzymes in the hydrolysis. Experiments were carried out using 12 differently pretreated lignocellulosic substrates. In most cases hemicellulases improved the degradation of polysaccharides. This positive effect was more pronounced when the residual hemicellulose content of the raw material was high. Sugar conversions increased, time of hydrolysis decreased compared to hydrolysis when only cellulases were applied.
Since the effect of hemicellulases was proved using purified enzymes, experiments were carried out using home made *T. reesei* enzymes, which were also compared to a commercial enzyme Celluclast 1.5L. The home made enzymes performed better in the hydrolysis, than the commercial one. This is probably due to the higher level of accessory (helper) enzymes in the own fermented enzyme complexes. The carbon source applied in the cultivation induced the production of cellulases and hemicellulases in proper quality and quantity.

6 Theses

I. The possibility to extract hemicellulose from corn fiber at high molecular weight (comparable to the traditional alkaline extraction) was proven. [*Benkő, Zs., Andersson, A., Szengyel, Zs., Gáspár, M., Réczey, K., Stålbrand, H. (2007) Heat extraction of corn fiber hemicellulose, Applied Biochemistry and Biotechnology, 136-140:253-265*]. Increasing the treatment temperature caused decrease of the molecular weight, but increase of the hemicellulose yield.

II. It was possible to produce thermoactive CBHI enzyme using the *T. reesei* RF6026 strain carrying the cbh1/cel7A gene of *Thermoascus aurantiacus*. With enzyme activity measurements I have proved that the mutant produces all other enzyme activities alike the parental strain. [*Benkő, Zs., Drahos, E., Szengyel, Zs., Puranen, T., Vehmaanperä, J., Réczey, K. (2007) Thermoascus aurantiacus CBHI/Cel7A production in Trichoderma reesei on alternative carbon sources, Applied Biochemistry and Biotechnology, 136-140:195-204*].

III. It was proven in lab scale fermentor experiments that the positive effect of tris-maleic acid buffer system is due to the presence of buffer components, and not only due to ensuring constant pH. [*Benkő, Zs., Réczey, K. (2007) Trichoderma reesei fermentációja alternatív szubsztrátokon, Műszaki Kémiai Napok'07, Veszprém, Műszaki Kémiai Kutató Intézet, ISBN: 978-963-9696-15-0, 62-66*].

IV. I have proved that application of xylanase and xyloglucanase enzymes as helper enzymes in the hydrolysis of lignocellulosic substrates improves the hydrolysis carried out by cellulases. While xyloglucanase improves the glucan conversion, xylanase improves the xylan conversion. The more remaining hemicellulose is in the raw material, the more pronounced is this effect. [*Benkő, Zs., Siika-aho, M., Viikari, L., Réczey, K. (2008) Evaluation of the role of xyloglucanase in the enzymatic hydrolysis of lignocellulosic substrates, Enzyme and Microbial Technology, 43:109-114, Benkő, Zs., Réczey, K. (2008) Lignocellulózok enzimes degradációja - A hemicellulázok, mint segítőenzimek, Magyar Kémikusok Lapja, 7-8:212-218*].

V. Home made enzymes performed better in the hydrolysis of complex (cellulose and hemicellulose containing) substrates, than the commercial Celluclast 1.5L applied in the same FPU dosage. [*Benkő, Zs., Tolvaj, B., Dienes, D., Réczey, K. (2008) Characterization of Trichoderma reesei Rut-C30 enzymes obtained on various carbon sources, Second Annual Workshop of COST FP0602, 4-5 of December, Biel, Switzerland*]. This is probably due to the higher level of helper enzymes in the home made enzyme complex.

7 Utilization

Biomass is a valuable, yearly renewable raw material, which could be used for biorefinery purposes. One of the most important biorefinery concepts is the production of bioethanol, which can substitute part of the fossil fuels. Nowadays, production is carried out using high starch containing plants (first generation biofuel), but since this is also the raw material of the food industry, it cannot be economically feasible in long term. This problem could be solved by application of lignocelluloses for production of ethanol (second generation biofuel). The technology required for that still exists only in lab and pilot scale. Therefore, there is great interest is in the research and development of this area. With my PhD work I wanted to contribute to this research. Taking into considerations the agricultural potential of Hungary, corn stover could be a relevant raw material of second generation bioethanol production. This is suitable for the production of enzymes, and also for the monosaccharides released by enzymatic hydrolysis. The enzyme produced on this raw material can be used for the hydrolysis without any concentration or purification step.

8 Publications

Scientific papers

I. Benkő, Zs., Andersson, A., Szengyel, Zs., Gáspár, M., Réczey, K., Stålbrand, H. (2007) Heat extraction of corn fiber hemicellulose, *Applied Biochemistry and Biotechnology*, 136-140:253-265. (IF-2007: 1,643)

II. Benkő, Zs., Drahos, E., Szengyel, Zs., Puranen, T., Vehmaanperä, J., Réczey, K. (2007) *Thermoascus aurantiacus* CBHI/Cel7A production in *Trichoderma reesei* on alternative carbon sources, *Applied Biochemistry and Biotechnology*, 136-140:195-204. (IF-2007: 1,643)

III. Benkő, Zs., Siika-aho, M., Viikari, L., Réczey, K. (2008) Evaluation of the role of xyloglucanase in the enzymatic hydrolysis of lignocellulosic substrates, *Enzyme and Microbial Technology*, 43:109-114. (IF-2007: 1,969)

IV. Benkő, Zs., Réczey, K. (2008) Lignocellulózok enzimes degradációja - A hemicellulázok, mint segítőenzimek, *Magyar Kémikusok Lapja*, 7-8:212-218.

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V. Gáspár, M., Benkő, Zs., Dogossy, G., Réczey, K., Czigány, T. (2005) Reducing water absorption in compostable starch-based plastics, *Polymer Degradation and Stability*, 90:563-569. (IF-2005: 1,749)

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Benkő, Zs., Siika-aho, M., Viikari, L., Réczey, K. (2007) Hemicellulázok hatása lignocellulózok enzimes degradációjára, XXX. Kémiai Előadói Napok Tudományos Szimpózium, Szeged, ISBN: 978-963-482-845-7, 126-130.

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Benkő, Zs., Siika-aho, M., Viikari, L., Réczey, K. (2008) Hemicellulases as helper enzymes in the degradation of lignocellulosic substrates, First COST Meeting, 27-28 of March, Copenhagen, Denmark

Benkő, Zs., Réczey, K., Viikari, L., Siika-aho, M. (2007) The role of xyloglucan hydrolysis in the total hydrolysis of lignocellulosic materials, North Carolina State University/ Helsinki area Institutes workshop, September, Helsinki, Finland
És: European Polysaccharide Network of Excellence (EPNOE) Scientific meeting, 7-8.11.2007, Iasi, Romania

Benkő, Zs., Siika-aho, M., Viikari, L., Réczey, K. (2007) How can xyloglucanase enhance the total hydrolysis of lignocellulosic substrates?, 29th Symposium on Biotechnology for Fuels and Chemicals, April 29 – May 2, Denver, CO, USA

Szijártó, N., Kádár, Zs., Benkő, Zs., Varga, E., Dienes, D., Réczey, K., (2006) Biorefinery Research at Budapest University of Technology and Econmics, European Conference on Biorefinery Research, 19 and 20 October 2006, Helsinki

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