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PRODUCTION AND CHARACTERIZATION OF NOVEL CELLULASE AND HEMICELLULASE ENZYMES BY SELECTED FILAMENTOUS FUNGI

Thesis of PhD dissertation

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1. Introduction

Diminishing resources of fossil-fuel energy and the detrimental effects of their usage on the environment has generated the growth of the application of plant biomass for fuel and chemical production (Ragauskas et al., 2006). First generation bioethanol plants (sugar cane and starch-based plant grains) are intensively studied, although their usage may increase the price of food and feed of animals. Therefore, the developing second generation biofuels produced from non-food biomass are also significant for future biorefineries (Sims et al., 2010). Source of second generation biofuel is raw materials such as energy plants and agricultural by-products e.g. wheat straw, corn stover and wood from agro- and forestry residues.

The present PhD research is part of the 7th EU Framework project, with the logo 'DISCO' ('Targeted DISCOvery of novel cellulases and hemicellulases and their reaction mechanisms for hydrolysis of lignocellulosic biomass', www.disco-project.eu). This project focuses on the development of efficient and cost-effective enzyme tools to degrade the lignocellulosic biomass to fermentable sugars, convert it to bioethanol and to investigate the performance of these enzymes.

The screening work was carried out approximately with 950 strains. Selected enzyme supernatants have been tested in hydrolysis experiments, in degradation of cellulose and hemicellulose rich carbon sources. Furthermore, performances of enyme mixtures were tested on different carbon sources in presence of different lignins. Sensitivity of enzymes was analyzed on different temperature and pH levels. Purification of enzyme preparation was performed and purified proteins were investigated.

Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA, Frederick Jr WJ, Hallett JP, Leak DJ, Liotta CL, Mielenz JR, Murphy R, Templer R, Tschaplinski T 2006. The path forward for biofuels and biomaterials. Science 311 (5760):484-489

Sims REH, Mabee W, Saddler JN, Taylor M. 2010. An overview of second generation biofuel techologies. Bioresource Techno. 101:1570-158

2. The Aims of This Thesis

- Discover and develop novel cellulase and hemicellulase enzymes that break down lignocellosic biomass more efficiently and more cost-effectively, making the bioethanol production process commercially viable in this respect. For this work we used TUB microbial culture collection (www.tub-collection.com).

- Investigation of novel hemicellulolytic fungal enzyme systems by a new screening method using up-to-date instruments, by which specific xylan degrading enzymes can be detected. The goal of screening selected lignocellulose degrading enzyme systems was to develop an improved hydrolysis of lignocellulosic biomass to fermentable sugars.

- Application of this screening method for a smaller subgroup of strains belonging to the *Trichoderma* section *Longibrachiatum* by diverse chromatography methods and a mass spectrometry method as well.

- Investigation of hydrolytic potential of novel *Penicillium* cellulases and new fast growing *Trichoderma* cellulases. The strains were cultivated on several lignocellulosic carbon sources and microcrystalline cellulose in order to produce efficient lignocellulose degrading enzyme supernatants.

3. Background

Lignocellulosic biomass represents a potential source of renewable raw materials for bioethanol production. Agricultural and forestry residues are a remarkable part of common solid waste (e.g. waste paper), as lignocellulosic materials are used as potential resources of biofuel production. The complex structure of biomass is made up of three important fractions: 35%–50% cellulose, 20%–35% hemicellulose and 12%–20% lignin. Furthermore, a minor amount of other polymeric components: starch, pectin, proteins, minerals, ash, etc (Gomez et al., 2008).

Efficient degradation of lignocellulosic biomass to oligo- and monosaccharides requires the cooperation of different microbial enzymes in synergism. Structurally two different enzymes are involved in the degradation of the plant cell wall, namely, endo-enzymes and exo-enymes. Endo-enzymes cut the bonds inside the chain of polymers. The action of endo-enzymes results in the reduction of substrate molecula mass. Exo-enzymes digest the endings of the polymer chain and hydrolyse oligomers to monomers. Endo-enzymes may provide additional substrates for the exo-enzymes (Henrissat et al., 1997).

Numerous protist and polycellular creature can produce lignocellulolytic enzymes although the most utilized lignocellulose digesting enzymes are originated from bacterias and fungi. The most deeply investigated enzyme systems include cellulases, hemicellulases and some connecting glycoside hydrolases (Himmel et al., 2010).

Up until now there has been not enough efficient enzyem cocktails for total degradation of the plant cell walls and the quantity of these enzymes are not known exactly. However, the usage of cellulase and hemicellulase enzyme mixtures may drive for a better and better degradation of the lignocellulosic biomass (Kristensen et al., 2008).

A great number of the industrial sector applies cellulase and hemicellulase enzyme mixtures, such as the food- and feed industry, pulp- and paper industry, textile- and laundry industry, baking industry, waste treatment or alcohol production from biomass.

Gomez LD, Steele-King CG, McQueen-Mason SJ. 2008. Sustainable liquid biofuels from biomass: the writing's on the walls. New Phytologist 178: 473–485

Henrissat B, Davies G. 1997. Structural and sequence-based classification of glycoside hydrolases. Current Opinion in Structural Biology. 7(5): 637–644

Himmel ME, Xu Q, Luo Y, Ding SY, Lamed R, Bayer EA. 2010. Microbial enzyme systems for biomass conversion: emerging paradigms. Biofuels. 1(2): 323-341.

Kristensen JB, Thygesen LG, Felby C, Jørgensen H, ElderNydetzky T. 2008. Cell-wall structural changes in wheat straw pretreated for bioethanol production. Biotechnol Biofuels. 1:5

Fungi play a crucial role in the degradation of the plant cell wall. They excrete extracellular enzymes, which together affect the plant cell wall materials releasing mono-, oligosaccharides and other cell wall components (Polizeli et al., 2005). Three classes of decaying fungi are distinguished according to which part of the lignocelluloses decay: white-rot, brown-rot and soft-rot fungi. White-rot fungi can degrade the lignin part of the plant matter, while brown-rot fungi are able to release the cellulose and hemicellulose part though not the lignin. Soft-rot fungi produce both polysaccharide- and lignin-degrading enzymes (Kirk et al., 1983).

Trichoderma species are some of the most abundant filamentous fungi in nature. They are well characterized and classified members of the Ascomycete brown-rot fungi group, which can be found in soils, on decaying wood and vegetable materials. All of these strains maintain the anamorph form of *Hypocrea* however, the *Hypocrea* teleomorph form exists only on wood or on other fungi, while strains of *Trichoderma* cause diseases of living plants as well and also mushroom. (Bailey et al., 1998).

Various lignocellulolytic enzyme systems of the *Penicillium* species have been investigated in the last 30 years (Liu et al., 2013). They produce high-effective cellulose degrading enzyme complexes, therefore they have improved the ability to degrade plant biomass.

Bailey BA, Lumsden RD. 1998. Direct efects of *Trichoderma* and *Gliocladium* on plant growth and resistance to pathogens, in *Trichoderma* & *Gliocladium*—Enzymes, G. F. Harman and C. P. Kubicek, Eds., vol. 2 of Biological Control and Commercial Applications, Taylor & Francis, London, UK. 327–342

Kirk TK. 1983. Degradation and Conversion of Lignocelluloses. In: Smith JE, Berry DR, Kristiansen B. The filamentous fungi, version 4, Fungal technology. London: Edward Arnold. 266-295

Liu G, Qui Y, Li Z, Qu Y. 2013. Improving lignocellulolytic enzyme production with Penicillium: from strain screening to systems biology. Biofuels. 4 (5): 523-534

Polizeli MLTM, Rizzatti ACS, Monti R, Terenzi HF, Jorge JA, Amorim DS. 2005. Xylanases from fungi: Properties and industrial applications. Appl Microbiol Biotechnol. 67: 577-591

4. Materials and methods

Microorganisms

Approximately 950 mesophilic filamentous fungi were tested in screening experiments. They were stored in our TUB (=Technical University of Budapest, www.tub-collection.com) culture collection and some of them were obtained from other culture collection (ATCC, NRRL, CBS, IFO, etc.). Isolation of lignocellulolytic fungi was performed on ground wheat straw and cellulose powder containing agar media. Two-three promising *Trichoderma* fungi from the *Longibrachiatum* section were found as good xylan degrading enzyme producers, while two *Penicillium* species could release novel cellulases. Two well-known *T. reesei* (RUT C30 and QM 6a) isolates were used as control in experiments.

Enzyme production and hydrolysis experiments

Shake flask fermentation was carried out in 750 mL Erlenmeyer flasks at 30°C, 220 rpm for 5 days. Measurement of colorimetric (traditional) enzyme activities and hydrolysis experiments were performed on different lignocellulosic materials by crude fermentation supernatants.

Analytical tools

Hydrolysis products (mono- and xylooligosaccharides) of soluble xylan rich substrates (wheat arabino-xylan, eucalyptus xylan hydrolysate) and insoluble xylan rich fractions (wheat straw water unextractable solids and corn fiber alcohol insoluble solids) digests were analyzed by HPAEC (High Performance Anion Exchange Chromatography), HPSEC (High Performance Size Exclusion Chromatography) and MALDI-TOF MS (Matrix Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry). Acetic acid content was measured by HPLC. This strategy is powerful in recognizing xylanases, arabinoxylan hydrolases, acetyl xylan esterases and glucuronidases.

5. Results

Testing of xylan degrading fungal enzymes on soluble hydrolysis substrates (Paper #1)

A new screening method using up-to-date methodology and instruments is described, by which specific xylan modifying enzymes can be detected. Fermentation supernatants of 78 different fungal soil isolates grown on wheat straw were used for hydrolysis of soluble xylan rich substrates (WAX, EXH). Hydrolysis products were analyzed by HPLC, HPAEC and MALDI-TOF MS. This strategy is powerful in recognizing xylanases, arabinoxylan hydrolases, acetyl xylan esterases and glucuronidases. No fungus produced all enzymes necessary to totally degrade the substrates tested. Some fungi produce high levels of xylanase active against linear xylan, but are unable to degrade complex xylans. Other fungi producing relative low levels of xylanase secrete many useful accessory enzyme components.

Degradation of insoluble wheat straw and corn fiber xylans by crude enzyme preparations (Paper #2)

Enzyme preparations of different fermentation supernatants were utilized in the hydrolysis of insoluble wheat straw and corn fiber xylan rich fractions (WS WUS and CF AIS). Up to 14% of the carbohydrates in wheat straw and 34% of those in corn fiber were hydrolyzed. The degree of hydrolysis by the enzymes depended on the origin of the fungal isolate and on the complexity of the substrate to be degraded. *Penicillium, Trichoderma* or *Aspergillus* species, and some non-identified fungi proved to be the best producers of hemicellulolytic enzymes for degradation of xylan rich materials. This study proves that the choice for an enzyme preparation to efficiently degrade a natural xylan rich substrates is dependent on the xylan characteristics and could not be estimated by using model substrates.

Characterization of xylan degrading enzymes of species belonging to the *Trichoderma* section *Longibrachiatum* (Paper #3)

Fifteen different strains from *Trichoderma* section *Longibrachiatum* have been tested for extracellular xylan degrading enzyme production on three carbon sources (wheat straw, corn fiber and eucalyptus wood) in shake flask cultivation. The enzyme activities were evaluated by traditional colorimetric enzyme assays and by novel instrumental methods (Figure 1).

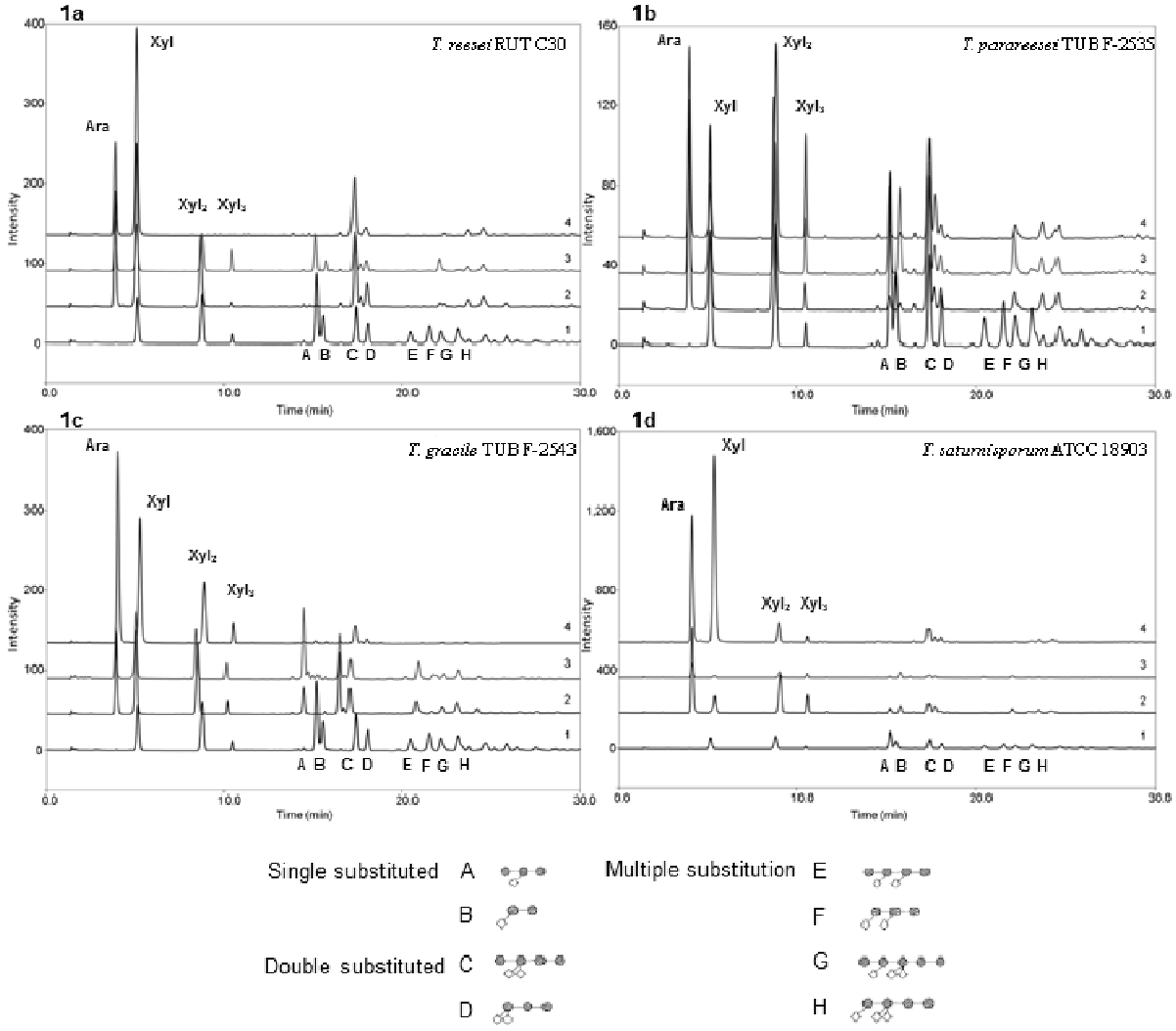

Figure 1. HPAEC profiles of hydrolysis product from digested wheat arabinoxylan (24h, 37°C, 500 rpm, pH:5.0) by four Trichoderma isolates fermented on three different fermentation carbon sources (wheat straw, eucalyptus wood, corn fiber). Line 1: endo-xylanase-I (reference); line 2: wheat straw fermentation supernatants; line 3: eucalyptus wood fermentation supernatants; line 4: corn fiber fermentation supernatants. Ara: arabinose; Xyl: xylose; Xyl_2: xylobiose; Xyl_3: xylotriose. 1a: T. reesei RUT C30; 1b: T. parareesei TUB F-2535; 1c: T. gracile TUB F-2543; 1d: T. saturnisporum ATCC 18903.

Hydrolysis of xylan was studied on four different xylan-rich model substrates (WAX, EXH, WS WUS and CF AIS). *T. reesei* CPK 155, *T. parareesei* TUB F-2535, *T. gracile* TUB F-2543 and *T. saturnisporum* ATCC 18903 isolates were equally good or better in degradation of the wheat arabinoxylan (WAX) and corn fiber alcohol insoluble solids (CF AIS) as hydolysis substrates than the well-known *T. reesei* QM 6a and RUT C30 strains. Therefore these fungi may be potential candidates for further experiments. Enzyme production on wheat straw and corn fiber carbon sources was more effective than on eucalyptus wood.

Hydrolysis of lignocellulosic materials by novel Penicillium cellulases (Paper #4)

The hemicellulolytic systems of two novel lignocellulolytic *Penicillium* strains (*P.pulvillorum* TUB F-2220 and *P.*cf.*simplicissimum* TUB F-2378) have been studied. The cultures of the *Penicillium* strains were characterized by high cellulase and β-glucosidase as well moderate xylanase activities compared to the *Trichoderma reesei* reference strains QM 6a and RUT C30. Comparison of the novel *Penicillium* and *T. reesei* secreted enzyme mixtures in the hydrolysis of (ligno)cellulose substrates showed that the F-2220 enzyme mixture gave higher yields in the hydrolysis of crystalline cellulose (Avicel) and similar yields in hydrolysis of pre-treated spruce and wheat straw than enzyme mixture secreted by the *T. reesei* reference strain. The sensitivity of the *Penicillium* cellulase complexes to softwood (spruce) and grass (wheat straw) lignins was lignin and temperature dependent: inhibition of cellulose hydrolysis in the presence of wheat straw lignin was minor at 35°C while at 45°C by spruce lignin a clear inhibition was observed. Therefore cellulase complex of *Penicillium pulvillorum* TUB F-2220 seems to be valuable in the simultaneous saccharification and fermentation (SSF) process performed at 35°C for pre-treated lignocelluloses. Purification of enzyme preparation was performed and purified proteins were investigated.

6. Thesis (new scientific results)

1. Novel screening method has been developed for investigation of various specific enzyme activities in crude fermentation supernatants. This method combines classical screening method together with different instrumental analytical tools such as High Performance Liquid Chromatography and Mass Spectrometry. (Paper #1)

2. This screening method is only effective when using the correct substrate. The substrate characteristics determine enzyme performance and screening results. Screening of xylan degrading enzymes was performed on soluble xylan rich substrates (wheat arabino-xylan, eucalyptus xylan hydrolysate) and insoluble xylan rich fractions (wheat straw water unextractable solids and corn fiber alcohol insoluble solids) Enzyme activity was more effective on soluble subtrates. (Paper #1 and #2)

3. Screening of 78 shake flask fermentation supernatants on different xylan rich substrates resulted in *Aspergillus*, *Penicillium*, *Trichoderma* and other non-identified species have high hemicellulolytic activity. Enzyme activity depends on origin of enzyme as well. These results could indicate further screening experiments of more strains. (Paper #2)

4. Screening of 15 species belonging to the *Trichoderma* section *Longibrachiatum* resulted in equally good or better isolates than control species *Trichoderma reesei* RUT C30 and QM6a. They are genetically close to *T. reesei* such as *T. reesei* CPK 155, *T. parareesei* TUB F-2535, and *T. gracile* TUB F-2543 isolates. They may represent a potential for industrial applications. (Paper #3)

5. Novel *Penicillium* cellulases were efficient in specific cellulase activity similarly to *Trichoderma reesei* RUT C30 cellulases. Also, higher β-xylosidase activity was investigated in *Penicillium* cellulase complex. The lignin inhibition of hydrolytic activity of the *Penicillium* cellulases was almost absent in case of herbaceous lignin being at a lower temperature (35°C). *P. pulvillorum* TUB F-2220 and *P. cf. simplicissimum* TUB F-2378 cellulases are competent in simultaneous saccharification and fermentation (SSF) at a lower temperature. (Paper #4)

7. Application of new scientific results

This study provides wide range of industrial applications:

- New screening analytical method may ensure novel xylan degrading enzymes.
- Testing of species genetically close to *Trichoderma reesei* (‘sister species’) may provides new potential for screening of non-identified isolates for production of cellulases and xylanases.
- Fast growing *Trichoderma* isolates may be efficient raw material for industrial production of cellulases. These results have not been published yet.
- The novel *Penicillium* cellulases may be apply in simultaneous saccharification and fermentation (SSF) at lower (35°C) temperature on herbaceous materials.

8. List of publications

1. Van Gool, M.P., Vancso, I., Schols, H.A., Szakacs, G., **Toth, K.,** Gruppen, H.: Screening for distinct xylan degrading enzymes in complex shake flask fermentation supernatants Bioresource Technology 102: 6039-6047 (2011) IF: 4.253

2. Van Gool, M.P., **Toth, K.,** Schols, H.A., Szakacs, G., Gruppen, H.: Performance of hemicellulolytic enzymes in a wide range of fermentation supernatants depends on substrate choice Bioresource Technology 114: 523-528 (2012) IF: 4.253

3. **Toth K.,** Van Gool M.P., Schols H.A., Samuels G.J., Gruppen H., Szakacs G.: Diversity in production of xylan degrading enzymes among species belonging to the *Trichoderma* section *Longibrachiatum*. Bioenergy Research 6(2): 631-643 (2013) IF: 3.562

4. Marjamaa K., **Toth K.,** Bromann P.A., Szakacs G., Kruus K.: Novel *Penicillium* cellulases for total hydrolysis of lignocellulosics. Enzyme and Microbial Technology. 52:358-369 (2013) IF: 2.367

Presentations

1. **Toth K.,** Van Gool M.P., Schols H.A., Samuels G.J., Gruppen H., Szakacs G.: Diversity in production of xylan degrading enzymes among species belonging to the *Trichoderma* section *Longibrachiatum*. 15th European Congress on Biotechnology, 23-26 September 2012, Istanbul, Turkey (poster presentation)

2. **Toth K.,** Van Gool M.P., Schols H.A., Szakacs G.: Hemicellulóz lebontó enzimek termelése és vizsgálata *Trichoderma* fonalasgomba fajokkal. XXXV. Kémiai Előadói Napok (KEN), 29-31 October 2012, Szeged, Hungary (oral presentation)

3. **Toth K.,** Van Gool M.P., Schols H.A., Szakacs G.: Xilanáz enzimek termelése és vizsgálata *Trichoderma* fonalasgomba fajokkal. MTA Természetes Polimerek Munkabizottsági Ülés, 28 November 2012, Budapest, Hungary (oral presentation)

4. **Toth K.,** Van Gool M.P., Schols H.A., Samuels G.J., Gruppen H., Szakacs G.: Xilán lebontó enzimek termelése és vizsgálata *Trichoderma* fonalasgomba fajokkal. X. Jubileumi Oláh György Doktori Konferencia, 07 February 2013, Budapest, Hungary (poster presentation)