Budapest University of Technology and Economics
Department of Applied Biotechnology and Food Science

PRODUCTION AND CHARACTERIZATION OF CELLULOSE-DEGRADING ENZYMES FOR VARIOUS APPLICATIONS

Ph.D. Thesis

Nóra Szijártó

Supervisor:
Dr. Kati Réczey

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1. INTRODUCTION AND AIMS OF THE PRESENT STUDY

Cellulose, the major structural polysaccharide of plant cell wall is by far the most abundant organic material on earth. Therefore enzymes able to degrade cellulose, or rather the microorganisms able to excrete such enzymes, play a substantial role in the global carbon cycle. Due to its physical properties, however, cellulose is a highly recalcitrant substrate for enzymatic degradability and the capacity to completely hydrolyze the cellulose macromolecule is restricted to a relatively select but diverse group of microorganisms. The most effective cellulase producers are found among the filamentous fungi (*e.g., Trichoderma, Aspergillus, Humicola, Melanocarpus*) and not surprisingly, industrial scale cellulase production basically relies on the use of these organisms.

Commercial production of cellulases by submerged fermentation - known to be superior to the solid state method to many aspects - began in the early 1970s, with cellulase made by *Trichoderma* sold for use in research and pilot studies. The mid 1980s saw the first large industrial application of cellulases and the use and demand for such enzymes has been rapidly increasing ever since. With their annual production of 23,000 tons and commercial sale of 125 million USD per year, cellulases are among the most important industrial enzymes today. At present they are widely used in food, brewery and wine, animal feed, textile and laundry, pulp and paper industries, as well as in research and development. However, predictions are serious that the most important application of cellulases requiring much larger quantities of these enzymes than they were in need ever before will be in the sustainable production of various chemicals, fuels, and other commodities starting from cheap cellulosic biomass as the feedstock. Bulk processes under discussion will, however, require much cheaper enzymes than those commercially available today.

According to some generally accepted benchmarks, the (cost-)efficient production of cellulases may require *(i)* a better understanding on the dynamics of cellulase secretion in relation with the quality and quantity of available carbon source as well as in relation with culture growth, so that to be able to improve the volumetric productivity during the fermentation process, *(ii)* the use of cheaper fermentation substrate and medium than those used traditionally, so that to decrease the feedstock cost known to stand for the largest portion of the total production costs, and *(iii)* the production of improved enzymes, so that to obtain products with special features or better hydrolytic properties.

In this view, the present work, which is a summary of six research papers as described later, was meant to provide a broadened view on the production and characteristics of various cellulose degrading enzymes from the (potential) application point of view. Therefore, it not only focuses on the most widely known and employed cellulase complex of the mesophilic soft rot fungus *Trichoderma reesei*, but also on the recently discovered cellulases of the thermotolerant ascomycete *Melanocarpus albomyces*. Covered fields involve: *(i)* the characterization of growth dynamics and enzyme secretion by a real cellulase producer in conventional and modified media using traditional and alternative fermentation substrates, *(ii)* heterologous production and purification of novel cellulases, and *(iii)* characterization of the produced enzymes by their activity, substrate specificity and hydrolysis potential, as the major topics.

2. MATERIALS AND METHODS

Materials

Chemicals used for the preparation of solutions and media were of analytical grade. A selection of cellulose preparations, lignocellulose derivatives, or other byproducts was used as the substrate of fermentation and hydrolysis experiments including: delignified pulp, Solka Floc (SF); Avicel; phosphoric acid swollen cellulose (PASC); old corrugated cardboard (OCC); the hydrolysed hemicellulose fraction wet-oxidized corn stover (WOCS hydrolyzate); the exhausted residue obtained after spirit or yeast manufacture on molasses (vinasses).

Cellulase enzyme fermentation by T. reesei

In cellulase production experiments *T. reesei* Rut C-30 was used as the test organism. Fermentations were carried out in shake flasks, a 3-l and a 30-l laboratory bioreactor using the basic mineral medium of Mandels together with 10 g/l of carbon source (28-30°C, pH 5-6, 200-350 rpm or 0.25 vvm). In specific cases the production medium was prepared by using WOCS hydrolyzate as a supplementary carbon source, vinasses as replacement for Mandels nutrients, or 0.1 M Tris-maleate buffer as a pH controlling agent.

Heterologous production and purification of M. albomyces cellulases

Genes derived from *M. albomyces* coding for the endoglucanase (EG) Cel45A and Cel7A, and the cellobiohydrolase (CBH) Cel7B (cellulases with different mode of action and site of attack on cellulose) were expressed in a genetically modified *T. reesei* deficient in its major native cellulases using lactose based complex nitrogen source medium. Genes were cloned in two variants: *(i)* using their native coding sequence, and *(ii)* with an exogenous cellulose binding module (CBM) sequence taken from a *T. reesei* CBH attached to the C-terminus of the native coding sequence. Enzymes were studied as culture filtrates after purification.

Hydrolysis of Avicel and PASC by M. albomyces cellulase

Hydrolytic properties of the *M. albomyces* cellulases applied individually and in controlled mixtures were studied using crystalline cellulose (Avicel) and amorphous cellulose (PASC) as the substrates (10 g/l). Hydrolysis reactions were carried out in 50 mM sodium phosphate buffer (pH 6.0) at 50°C with magnetic stirring in Eppendorf tubes at 1.8 ml volume. Enzymes were loaded as proteins in a dosage of 5, 10, 20 mg/g and 0.5, 1, 2, 5 mg/g protein per substrate when Avicel and PASC was used, respectively. Hydrolysis reactions were carried out individually for each time point.

Determination of cellulase activities

To describe the overall (absolute, non-specific) potential of the produced cellulase complex to hydrolyze cellulose, filter paper degrading activity (FPA) of samples taken from shake flask and bioreactor cultures has been determined. EG activity of the *M. albomyces* cellulases was measured against hydroxyethyl cellulose (HEC) and carboxymethyl cellulose (CMC). The β-glucosidase activity of enzymes was determined against *p*-nitrophenyl-β-d-glucopyranoside.

3. NEW SCIENTIFIC RESULTS

This study reports investigations on *(i)* the production of cellulose degrading enzymes by *Trichoderma reesei* most commonly employed to produce cellulases for commercial purpose at industrial-scale, and *(ii)* the hydrolytic properties of three recently discovered cellulases from *Melanocarpus albomyces* ideally suited for various applications but not yet discovered by the relevant industries.

The contribution of the present report to the existing knowledge on cellulose degrading enzymes can be summarized briefly as follows.

1. A two-stage process for the cultivation of *T. reesei* Rut C-30 using glucose as the carbon source in the first stage and cellulose in the second stage was found to be suitable to study the secretion of cellulases by non-induced cells as the response to pulse addition of their natural substrate. In this manner valuable information on the dynamics and enzyme production by the cultured cells can be obtained. This study is the first to report that *T. reesei* Rut C-30 is able to switch its metabolism from glucose consumption to cellulose utilization in an immediate manner. This ability, taking into account the numerous advantages that soluble substrates have over solid substrates, is foreseen to be well exploited in process development.
   (Paper I, Paper II)

2. Fermentation performance of *T. reesei* Rut C-30 as evaluated via produced cellulase activity is substantially (up to 43%) improved when cells used to initiate growth in the production medium are pre-grown on an inducing substrate (SF) instead of a non-inducing one (glucose). This study is the first to report exact data for such improvement.
   (Paper I)

3. This study is the first to evaluate the nitrogen content of Mandels medium, which was found to be approx 10% higher than the real nitrogen uptake by the cultivated *T. reesei* Rut C-30 in the same medium, when all nitrogen is supplied as inorganic substance.
   (Paper I)

4. This study is the first to employ hydrolyzed hemicellulose obtained after wet-oxidation of corn stover as a supplementary carbon source during cellulase production by *T. reesei* Rut C-30, which was proven to be suitable for such purpose. In particular, the hydrolyzate derived from wet-oxidation performed at 185°C for 5 min under alkaline conditions improves fermentation performance by 10% over the control. The use of 0.1 M Tris-maleate is convenient to control such cultures at pH 6.0. (Paper III, Paper IV)

5. Used paper material is a suitable substrate for cellulase production by *T. reesei* Rut C-30. Diluted vinasses can be used as the sole nutrient source in such experimental cultivations to replace Mandels nutrients. (Paper V)

6. This study is the first time to investigate the hydrolytic properties of Cel7A, Cel7B, and Cel45A of *M. albomyces*. The studied enzymes are less active on crystalline cellulose than on amorphous cellulose. The studied CBH has greater activity than the EGs against crystalline substrate, whereas in the case of amorphous substrate the order is reversed. Evidence of synergism can be seen when the enzymes are applied in mixtures. Addition of an exogenous CBM to the enzymes originally lacking it improves their hydrolytic potential; it has a greater effect on the EGs than the CBH, especially against crystalline substrate. The tested CBH was more effective on amorphous cellulose than the corresponding enzyme from *T. reesei* already at pH 6.0, 50°C. Due to their increased thermostability and tolerance of non-physiological pH, novel enzymes are expected to perform even better at higher T and pH. (Paper VI)

4. LIST OF PUBLICATIONS

This study is based on the following papers:

I. **Szijártó, N.**, Lidén, G., Réczey, K. Effect of medium nitrogen content on cellulase production by *Trichoderma reesei*. Manuscript before submission.

II. **Szijártó, N.**, Szengyel, Zs., Lidén, G., Réczey, K. (2004) Dynamics of cellulase production by glucose grown cultures of *Trichoderma reesei* Rut C-30 as a response to addition of cellulose. *Appl. Biochem. Biotechnol.* 113-116, 115-124. (IF: 0.805)

III. Juhász, T., Szengyel, Zs., **Szijártó, N.**, Réczey, K. (2004) The Effect of pH on the cellulase production of *Trichoderma reesei* Rut C-30. *Appl. Biochem. Biotechnol.* 113-116, 201-211. (IF: 0.805)

IV. **Szijártó, N.**, Varga, E., Réczey, K., Thomsen, A.B. Cellulase enzyme production by *Trichoderma reesei* Rut C-30 on the separated liquid stream from wet oxidation of corn stover. Submitted for publication to *Enzyme Microbiol. Technol.* (IF: 1.705)

V. **Szijártó, N.**, Faigl, Zs., Mézes, M., Bersényi, A., Réczey, K. (2004) Cellulase fermentation on a novel substrate (waste cardboard) and subsequent utilization of home-produced cellulase and commercial amylase in a rabbit feeding trial. *Ind. Crops Prod.* 20, 49-57. (IF: 0.746)

VI. **Szijártó, N.**, Siika-aho, M., Tenkanen, M., Alapuranen, M., Vehmaanperä, J., Réczey, K., Viikari, L. Hydrolysis of amorphous and crystalline cellulose by heterologously produced cellulases of *Melanocarpus albomyces*. Submitted for publication to *J. Biochem*. (IF: 1.827)

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Szijártó, N., Varga, E., Réczey, K., Thomsen, A.B. Integrated process scheme for on-site production of cellulose degrading enzymes in the lignocellulose to ethanol process using wet oxidised corn stover as the substrate. *14th European Conference and Technology Exhibition on Biomass for Energy, Industry and Climate Protection*. Paris, France, October 17-21, 2005, pp. 1851-1854.

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