PRODUCTION OF CELLULOLOYTIC ENZYMES WITH TRICHOSTERMA ATROVIRIDE MUTANTS FOR THE BIOMASS-TO-BIOETHANOL PROCESS

Cellulase Production, Enzymatic Hydrolysis and Simultaneous Saccharification and Fermentation

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1. INTRODUCTION, AIM OF THE THESIS

The price of commercial enzymes used for the saccharification of pretreated lignocellulosic materials represents a significant part (approximately 10%) in the overall cost of the biomass-to-bioethanol process. Reduction of the production cost, hence development of more effective cellulase-secreting microorganisms and improvement of the hydrolytic properties of enzyme mixtures are of great importance.

*Trichoderma reesei* has been chosen by many researchers and industrial companies to produce commercial cellulases in the last four decades, even though this species practically does not secrete β-glucosidase, a key enzyme for the complete hydrolysis of cellulose, into the liquid phase. Therefore, the enzymes of *T. reesei* have to be supplemented with extra β-glucosidase preparations in order to reach high glucose yield in the saccharification of cellulose.

The aim of this thesis was to develop new *Trichoderma* mutants that produce more efficient cellulolytic enzymes for the saccharification of pretreated lignocellulosic materials than *T. reesei* Rut C30. In order to reduce the production cost of bioethanol, various process streams from the ethanol production process, such as steam-pretreated willow (SPW), spruce (SPS), wheat straw (SPWS) and sugarcane bagasse (SPB), were used as substrates for enzyme production. The crude enzymes were characterized by various enzyme activity assays.

The main objectives of the studies were to achieve higher glucose concentration in enzymatic hydrolysis and higher ethanol yield in simultaneous saccharification and fermentation (SSF) of pretreated biomass with enzymes produced in-house by the new *Trichoderma* mutants than with *T. reesei* enzymes or commercial preparations.
The experimental part of the thesis consisted of five main steps:

1) Screening and selection of an appropriate wild-type Trichoderma strain for cellulase production on steam-pretreated lignocellulosic substrates
2) Development of Trichoderma mutants by UV-irradiation and chemical mutagenesis
3) Production of cellulolytic enzymes with the most promising mutants on steam-pretreated substrates in shake flask fermentation and in lab-scale fermentors in comparison with the well-known mutant T. reesei Rut C30
4) Evaluation of the hydrolytic potential of the enzymes produced in-house by the mutants in comparison with commercial enzyme preparations
5) Investigation of the efficiency of the produced enzymes in SSF to produce ethanol from biomass

The work was carried out in collaboration between the Department of Applied Biotechnology and Food Science (BME, Hungary) and the Department of Chemical Engineering (LU, Sweden).
2. EXPERIMENTAL METHODS

2.1. Production of Trichoderma mutants

*Trichoderma* mutants were developed by UV-irradiation and chemical mutagenesis. Good cellulase-producing mutant colonies showed clearing zones on plates containing phosphoric-acid-swollen cellulose.

2.2. Enzyme production

Various media containing steam-pretreated lignocellulosic substrates were used in shake flasks and fermentors (2-L Labfors) to investigate cellulase and β-glucosidase production of *Trichoderma* mutants in comparison with *T. reesei* Rut C30. Cellulase activity was measured on Whatman No. 1 filter paper (filter paper activity: FPA) and β-glucosidase activity was assayed using p-nitrophenyl-β-D-glucopyranoside as substrate.

2.3. Enzymatic hydrolysis of pretreated lignocelluloses

Hydrolysis was performed on washed or unwashed steam-pretreated lignocellulosic substrates. When comparing the performance of the enzymes produced in-house to that of commercial preparations, enzyme loadings were based on equal FPA/substrate dosage using Celluclast 1.5L, Novozym 188 and Accellerase™ 1000 as references. When comparing the efficiency of the various crude enzymes produced in-house, the same amount of produced enzyme (supernatant or whole fermentation broth)/substrate dosage was employed.

2.4. Simultaneous saccharification and fermentation

In order to investigate whether the crude *T. reesei* Rut C30 and *T. atroviride* F-1663 enzymes produced in-house can be directly applied in SSF of SPS, ethanol production was carried out in 1-L shake flasks, using *Saccharomyces cerevisiae* to ferment the sugars to ethanol. The enzymes produced were used at a loading of 10 g enzyme solution (supernatant or whole broth) per g WIS substrate (WIS: water-insoluble solids).

To compare the in-house-produced enzymes with commercial cellulases, the SSF experiments were performed in 2-L fermentors. The performance of the concentrated enzyme supernatants of Rut C30 and F-1663 was compared to that of commercial enzymes (3:1 mixture of Celluclast 1.5L&Novozym 188, Accellerase™ 1000) at 5 FPU/g WIS.
3. RESULTS

3.1. Enzyme production with T. atroviride strains

*T. atroviride* F-1505 proved to be the most promising extracellular cellulase producer among more than 150 wild-type *Trichoderma* strains in a screening program performed in shake flask fermentation on SPS and SPW, and was therefore selected as the parent strain for mutagenesis.

The new *T. atroviride* mutants, developed from F-1505 with UV-irradiation and chemical mutagenesis, proved to have excellent extracellular β-glucosidase activities and grew faster on pretreated lignocellulosic substrates than the control strain *T. reesei* Rut C30. Furthermore, the mutants produced significantly higher cellulase and β-glucosidase activities than the parent strain both in shake flask fermentation and in fermentors. However, their maximal FPA was about 5-30% lower than that of Rut C30.

3.2. Enzymatic hydrolysis of steam-pretreated lignocellulosic substrates

Due to high levels of extracellular β-glucosidase activities, enzyme supernatants of the *T. atroviride* strains hydrolyzed the pretreated lignocellulosic substrates to glucose more efficiently than the supernatant of *T. reesei* Rut C30.

Enzymes bound to the mycelia or to the lignocellulose residues proved to play an important role in the hydrolysis of SPS, especially in the case of Rut C30. Application of the whole fermentation broths instead of the supernatants resulted in an improvement in the final glucose concentration of about 200% and 15% for Rut C30 and the *T. atroviride* strains, respectively (Fig. 1). These results suggested that the *T. atroviride* strains mostly produced free extracellular enzymes, while Rut C30 produced high amount of mycelium-bound enzymes.

Due to bound enzymes, the use of the washed fermentation solids of *T. reesei* and the *T. atroviride* mutants as supplements to the low-β-glucosidase-containing Celluclast 1.5L, proved to improve the hydrolytic performance of this commercial preparation by 90-155%.

Hydrolytic performance of the enzymes produced in-house by the mutant *T. atroviride* F-1663 was equal to that of commercial cellulases on each pretreated lignocellulosic substrates investigated in terms of the glucose produced at same FPU/g WIS enzyme dosage.
3.3. SSF of SPS using enzymes produced in-house

In accordance with the results obtained in the enzymatic hydrolysis, the application of the whole broths of *T. atroviride* F-1663 and *T. reesei* Rut C30 enhanced the final ethanol yields in SSF compared to when only the supernatants were used. Greater improvement was observed in the case of the Rut C30 enzymes, where the use of the whole broth instead of the enzyme supernatant increased the final ethanol yield from 36% to 61% of the theoretical (based on the glucan, glucose and mannose content of SPS). However, the highest ethanol yield (66% of the theoretical) was obtained with the whole fermentation broth of *T. atroviride*.

The concentrated supernatants of *T. reesei* Rut C30 and *T. atroviride* F-1663 were compared to commercial cellulases in SSF of SPS in lab-scale fermentors. The highest ethanol yield (76% of the theoretical) was achieved with the *T. atroviride* enzyme preparation. Ethanol yields of about 70% of the theoretical were obtained with the commercial enzymes, whereas low ethanol yield (<35% of the theoretical) was obtained with the β-glucosidase-deficient *T. reesei* supernatant (Fig. 2).
Figure 2. Ethanol concentrations and yields (% of the theoretical) in SSF of unwashed SPS in 2-L fermentors using the 3:1 mixture of Celluclast 1.5L & Novozym 188, Accellerase™ 1000, and the concentrated enzyme supernatants of *T. atroviride* F-1663 and *T. reesei* Rut C30 produced on SPS. The theoretical maximum ethanol concentration was 23.7 g/L. (T: 35°C, 96h, pH 5, substrate concentration: 50 g/L WIS, yeast concentration: 2 g DM/L, enzyme loading: 5 FPU/g WIS)
4. NEW SCIENTIFIC FINDINGS

1. Good extracellular cellulase- and β-glucosidase-producing mutants were developed from the wild-type strain *Trichoderma atroviride* F-1505 (*Paper I*).

2. Due to high levels of extracellular β-glucosidase activities, the enzyme supernatants produced in-house by the new *T. atroviride* mutants proved to have better hydrolytic potentials than the *T. reesei* Rut C30 supernatants and the commercial Celluclast 1.5L preparation on steam-pretreated willow (SPW) (*Paper I*) and spruce (SPS) (*Papers II and III*).

3. The *T. atroviride* cellulases produced in-house proved to have about 10°C lower temperature optima than the Rut C30 enzymes for both short- and long-term hydrolysis (*Paper II*). This fact can be beneficial in simultaneous saccharification and fermentation (SSF).

4. The use of the whole fermentation broth of *T. reesei* Rut C30, containing both the free and the bound enzymes, significantly improved the glucose yield in enzymatic hydrolysis (*Paper II*) and the ethanol yield in SSF (*Paper III*) compared to the fermentation supernatant. This suggested that *T. reesei* had high amount of mycelium-bound β-glucosidase enzymes, whereas the *T. atroviride* strains mostly secreted free extracellular β-glucosidases.

5. The enzymes produced in-house by the mutant *T. atroviride* F-1663 proved to be more efficient than commercial preparations in SSF of SPS at equal FPA/substrate loadings (*Paper III*).

6. Due to the low ratio of β-xylosidase to xylanase activity, the supernatants produced by *T. atroviride* F-1663 on various steam-pretreated lignocellulosic substrates hydrolyzed the xylan and the xylose oligomers found in steam-pretreated wheat straw and sugarcane bagasse less efficiently than the commercial preparation tested. It was shown, that while *T. reesei* wild-type strains and mutants produce low levels of extracellular β-glucosidase, the new *T. atroviride* mutants secrete suboptimal quantities of β-xylosidase (*Paper IV*).
5. CONCLUSION

New enzymes, produced on-site on cheap lignocellulosic substrates by a *Trichoderma* strain with good FPA and high extracellular β-glucosidase activity (e.g. *T. atroviride* mutants) or enzyme preparations that also contain the cell-wall-bound β-glucosidase of *T. reesei*, could lead to more cost-effective production of second-generation bioethanol.

The new *T. atroviride* mutants described in this thesis have the advantage of producing high levels of extracellular β-glucosidase activities, and may be potential strains of choice for the production of cellulases. The enzymes produced by the mutant *T. atroviride* F-1663 were found to be somewhat more efficient than commercial enzymes in the enzymatic hydrolysis and the production of ethanol by SSF of SPS. On the other hand, the mutant had the drawback of secreting suboptimal levels of β-xylosidase enzymes, leading to inefficient xylan conversion. However, the strain could be further improved by additional mutation experiments. Alternatively, more severe pretreatment conditions could be employed to break down the xylan content of the lignocellulosic substrates more efficiently, rendering the addition of β-xylosidase in enzymatic hydrolysis unnecessary.

In the future, further improvement of the well-known cellulolytic strains and their enzyme complexes, optimization of the technology of enzyme production and screening for new cellulolytic microorganisms, could make lignocellulose hydrolysis, the bottleneck of the biomass-to-bioethanol process these days, more efficient.
6. LIST OF PUBLICATIONS

Papers on which the thesis was based


Other publications


Oral presentations and posters


Kovacs K, Megyeri, L, Szakacs, G, Galbe, M, Zacchi, G. Fermentation and hydrolysis experiments with new Trichoderma mutants in comparison with Trichoderma reesei Rut C30. 9th International Workshop on Trichoderma and Gliocladium, Vienna, Austria, Apr. 6-8, 2006. (poster)

Kovacs K, Megyeri, L, Szakacs, G, Chipeta, Z, Christopher, L. Production of xylanase with low cellulase activity on eucalyptus soda aq pulp in solid state fermentation by Trichoderma strains. 9th International Workshop on Trichoderma and Gliocladium, Vienna, Austria, Apr. 6-8, 2006. (poster)


Kovacs, K, Szakacs, G, Pusztahelyi, T. Production of chitinolytic enzymes with Trichoderma longibrachiatum IMI 92027 in solid substrate fermentation. 8th International Workshop on Trichoderma and Gliocladium, Hangzhou, China,


