

**Ph.D. thesis**

**SOLID STATE FERMENTATION  
AND UTILIZATION OF XYLANASE  
IN PULP BLEACHING**

AUTHOR  
**JUDIT SZENDEFY**

SUPERVISOR  
**DR. GYÖRGY SZAKÁCS**  
CONSULTANT  
**PROF. LEW CHRISTOPHER**

BUDAPEST UNIVERSITY OF TECHNOLOGY AND ECONOMICS  
DEPARTMENT OF AGRICULTURAL CHEMICAL TECHNOLOGY  
BUDAPEST, 2005

## **1. INTRODUCTION, AND GOALS**

Conventionally chlorine is used for the bleaching of paper pulp which results in washable chlor-lignin from lignin and chlor-phenols from the degradation products of lignin. The chlorinated compounds (including dioxins) present in wastewater and the wastewater sludge generated during bleaching have a high impact on the environment, therefore the introduction of environmentally friendly bleaching processes to replace chlorine fully or partially has become necessary. Chemical bleaching supplemented by xylanase pretreatment (biobleaching) is currently the only biotechnological solution for the bleaching of pulp that is also implemented in the industry. The paper industry requires cellulase-free xylanase products, or products with a low cellulase content, since cellulose present next to the xylanase may deteriorate the strength of pulp during biobleaching.

The enzymes commercially available for biobleaching are produced by Submerged Fermentation (SF) usually followed by the extraction of the enzyme and its purification before use. Solid State Fermentation (SSF) is a cost-effective option for the production and utilization of many enzymes.

The foremost aim of my Ph.D. research work was the production of xylanase by SSF that can be used for the biobleaching of paper pulp and contains only a negligible amount of cellulose. I have investigated the possibility of directly utilizing the enzyme-rich,

untreated fermentation product for biobleaching, thereby, possibly reducing the treatment costs significantly. The research work has been carried out in collaboration with the Biotechnology Laboratory of the South African Pulp and Paper Industry (SAPPI).

## **2. EXPERIMENTAL METHODS**

### **2.1 Solid State Fermentation**

Filamentous fungi were cultivated in 500 ml Erlenmeyer flasks on the substrates that were subsequently used for biobleaching, namely eucalyptus pulp and bagasse (the residual of sugar cane extraction) pulp. Thereby, we ensured the induction of a substrate specific enzyme complex.

### **2.2 Statistical experimental methods/designs**

A 2-level fractional factorial design and the response surface method was used for the optimization of xylanase production. The computer program Statistica for Windows 2000 (StatSoft, Inc.) was used for the construction and evaluation of the experimental designs.

### **2.3 Biobleaching**

Xylanase treatment preceding chemical bleaching was carried out with the raw fermentation products at different SSF material:pulp mass ratios (1/50-1/400), or with the addition of 5 IU/g of the commercial enzyme product. The brightness of the treated pulp samples was measured by the directional reflectance at 457 nm.

### 3. SUMMARY OF NOVEL SCIENTIFIC RESULTS

The novel results of the research work presented in my Ph.D. thesis can be summarized as follows:

- [1] I have confirmed that through the cultivation of *Thermomyces lanuginosus* and *Aspergillus oryzae* strains by solid state fermentation on either eucalyptus or bagasse pulp, the fermentation product can be directly applied for the biobleaching of the pulp used for fermentation without the need for extraction and purification of the xylanase. The products of the best performing strains have demonstrated biobleaching results identical to those of the commercially available (SF) enzymes.
- [2] I have determined that the *T. lanuginosus* strains generally produce more xylanase than the *A. oryzae* strains on the examined substrates (eucalyptus and bagasse pulp), however the the xylanases from *A. oryzae* are more effective during biobleaching.
- [3] By optimizing the xylanase production in SSF of the most effective strains (*T. lanuginosus* TUB F-980, *A. oryzae* NRRL 3485, *A. oryzae* NRRL 1808), I have confirmed that nitrogen addition is a requirement for high level xylanase production in the case of both eucalyptus and bagasse pulp.

By investigating the effect of inorganic and organic nitrogen sources (Potassium nitrate, Ammonium nitrate and Ammonium sulphate; corn steep liquor (CSL) and defatted soybean meal) on xylanase production, both independently and in combination with each other using a fractional factorial design, I have proved that the nitrogen sources influence each others effects. By optimizing xylanase production using the response surface method I have verified that, due to the existing interactions, the addition of two types of nitrogen source at given ratios results in significantly higher xylanase production rates than just increasing the amount of one type of nitrogen source.

[4] I have verified that the pH during SSF can be controlled by changing the amounts and the ratio of the two different nitrogen sources, thereby xylanase yield is also increased by the ideal pH value for enzyme production fixed by the optimized nitrogen source.

A disadvantage of SSF as compared to submerged fermentation is that pH control in the fermentor is unresolved, therefore it is especially important that through the quantitative and qualitative modification of the nitrogen source the pH value of fermentation can be influenced.

- [5] By investigating the production of accessory enzymes ( $\beta$ -xylosidase,  $\alpha$ -arabinofuranosidase and  $\alpha$ -galaktosidase) presumably playing a role in effective biobleaching, which are produced next to xylanase by *A. oryzae* NRRL 1808 strain, I have determined that similar parameter values favour the production of the associated enzymes and xylanase on bagasse pulp with regard to nitrogen requirement and starting pH values, therefore the yields on this substrate can be increased simultaneously
- [6] I have shown that the optimal pH and temperature values of the xylanases produced by the best performing *A. oryzae* strains (NRRL 3485, NRRL 1808) in SSF (pH 6.5 and 65 °C in both cases) are higher than the characteristics values of the *A. oryzae* xylanases described earlier in literature. Based on the measurements of thermal stability of the enzyme in the extract and in the presence of the SSF material I have confirmed that the SSF material inhibits the thermal inactivation of the enzymes. In its presence both xylanases are stable at 50 °C for one hour. The detailed parameters presumably play a role in the effective biobleaching effect of the two *A. oryzae* xylanases.

[7] I have demonstrated that in the case of the most effective xylanases during biobleaching (xylanases of *T. lanuginosus* TUB F-980, *A. oryzae* NRRL 3485 and *A. oryzae* NRRL 1808) as a result of 1,5-2-fold increase in xylanase yield using the optimized medium, the enzyme charge on pulp increased proportionally which resulted in enhancement in the bleaching performance of the raw SSF materials. A reduction of 25-35 % in chlorine dioxide charges was reached during biobleaching of eucalypts pulp with optimized SSF materials, demonstrating brightness identical to those of the only chemical bleaching, which proves the high productivity of biobleaching.

#### **4. APPLICATION POSSIBILITIES**

The experimental results have opened several doors to achieving the industrial implementation of alternative biobleaching using SSF enzymes. Unlike submerged fermentation, SSF does not require intense mixing and aeration, cultivation is carried out in a simple equipment, furthermore, in the biobleaching process that we have developed the xylanases do not need to be separated and cleaned. Therefore, the raw SSF products rich in xylanase provide the same brightness as the commercial (SF) enzymes, but at significantly lower enzyme treatment costs. The presence of SSF material makes it possible for enzymes that become heat-sensitive following extraction to be used for biobleaching.

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## 5. PUBLICATIONS ON THE SUBJECT OF THE THESIS

### 5.1 Journal papers

1. Christopher, L., Bissoon, S., Singh, S., Szendefy, J., Szakacs, G., Bleach-enhancing abilities of *Thermomyces lanuginosus* xylanases produced by solid state fermentation. *Process Biochem.* **40**, **2005**, 3230-3235.
2. Szendefy, J., Szakacs, G., Christopher, L. Potential of solid-state fermentation enzymes of *Aspergillus oryzae* in biobleaching of paper pulp. (2005). Submitted to *Enzyme and Microbial Technology*.
3. Szendefy, J., Szakacs, G., Christov, L. Biobleaching efficiency of *Aspergillus oryzae* xylanases produced by solid substrate fermentation. In Lignocellulose Biodegradation, B.C. Saha & K. Hayashi, eds, *ACS Symp. Ser. 889*, American Chemical Society, Washington, DC, **2004**, 316-338.
4. Szendefy, J., Szakacs G., Kemeny S., Christov, L. *In situ* solid-state fermentation and utilization of xylanase in pulp bleaching. In Applications of Enzymes to Lignocellulosics, S.D.Mansfield & J.N. Saddler, eds, *ACS Symp. Ser. 855*, American Chemical Society, Washington, DC, **2003**, 255-284.

5. Szendefy, J., Szakács, G., Christov, L. Xilanáz enzimek termelése termofil fonalagombákkal szilárd fázisú fermentációban. *Acta Biol. Debrecina* **2000**, 129-134.

## 5.2 Oral presentations

1. Szendefy, J., Szakács, G. and Christov, L. Potential of *Aspergillus oryzae* xylanases produced by solid substrate fermentation for biobleaching of pulp, **ACS Symposium on Advances in Biodegradation and Biotransformation of Lignocellulosics**, **2003**, March 23-27, New Orleans, LA, USA
2. Szendefy, J., Szakács, G., Kemény, S. and Christov, L. *In-situ* solid-state fermentation and utilization of xylanase in pulp bleaching. **ACS Symposium on Xylans, Mannans and other Hemicelluloses**, American Chemical Society, **2002**, Apr. 7-11, Orlando, Florida
3. Szakács, G., Bogár, B., Szendefy, J., Christov, L. and Tengerdy, R.P. Production of alpha-amylase and xylanase by solid substrate fermentation. **AMI Symposium on Microbial Biotechnology**, Association of Microbiologists of India, Trivandrum Chapter, **2002**, Jan.16, Trivandrum, India

4. Szakács, G., Bogár, B., Szendefy, J., Christov, L. and Tengerdy, R.P. Production of alpha-amylase and xylanase by solid substrate fermentation. **Pacificchem, 2000**, Dec.14-19, Honolulu, Hawaii

### **5.3 Poster presentations**

1. Szakacs, G., Szendefy, J., Christov, L. Biobleaching efficiency of thermostable fungal xylanases produced by solid state fermentation. **9<sup>th</sup> International Conference on Biotechnology in the Pulp and Paper Industry, 2004**. Oct. 10-14, Durban, South Africa
2. Szendefy, J., Szakács, G. and Christov, L. Screening of thermophilic fungi in solid state fermentation for xylanase production, **International Conference on the Emerging Frontiers at the Interface of Chemistry and Biology (ICB-2003), 2003**, Apr. 28-30, Trivandrum, India
3. Szendefy, J., Szakács, G. and Christov, L. Production of xylanase by solid substrate fermentation. **II. Magyar Mikológiai Konferencia, 2002**, Máj. 29-31, Szeged
4. Maré, J.E., Szendefy, J., Szakács, G., du Preez, J.C. and Christov, L. Isolation and evaluation of xylanases from thermophilic and

thermotolerant microorganisms in pulp bleaching. **8<sup>th</sup> International Conference on Biotechnology in the Pulp and Paper Industry**, 2001, June 4-8, Helsinki, Finland

5. Szakács, G., Szendefy, J. and Christov, L. Screening of thermophilic fungi in solid state fermentation for xylanase production. **International Conference on New Horizons in Biotechnology**, 2001, Apr. 18-21, Trivandrum, India
  
6. Szendefy, J., Szakács, Gy. és Christov, L. Xilanáz enzim termelése termofil fonalagombákkal szilárd fázisú fermentációban. **IX. Fermentációs Kollokvium**, 2000, Okt. 5-8, Debrecen

## **6. PUBLICATIONS NOT DIRECTLY RELATED TO THE SUBJECT OF THE THESIS**

### **6.1 Oral presentations**

- 1 Szendefy, J., Bényi, T., Domonkos, D., Szakács, G. and Csiszár, E. Termofil fonalagombák vizsgálata indigóval színezett farmerszövetek kőmosást helyettesítő enzimes kezelésére. **XXII. Kémiai Előadói Napok, 1999**, November 1-3, Szeged.
2. Szakács, G., Tengerdy , R. P., Urbánszki, K., Szendefy, J., and Halász, D. Comparison of corn fiber hydrolysis by a crude solid substrate fermentation enzyme and Celluclast 1.5L. **Recent Advances in Fermentation Technology (RAFT III), 1999**, Nov. 13-16, Sarasota, Florida, USA