Fungal Glucuronoyl and Feruloyl Esterases for Wood Processing and Phenolic Acid Ester/Sugar Ester Synthesis

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INDUSTRIAL BIOTECHNOLOGY

Introduction

Feruloyl esterases (FAEs, E.C. 3.1.1.73, CAZy family CE1) and glucuronoyl esterases (GEs, E.C. 3.1.1.-, CAZy family CE15) are involved in the degradation of plant biomass by hydrolysing ester linkages in plant cell walls, and thus have potential use in biofuel production from lignocellulosic materials and in biorefinery applications with the aim of developing new wood-based compounds [1, 2]. GEs and FAEs are present in the genomes of a wide range of fungi and bacteria.

Under conditions of low water content, these enzymes can also carry out (trans)esterification reactions, making them promising biocatalysts for the modification of compounds with applications in the food, cosmetic and pharmaceutical industry. Compared to the chemical process, enzymatic synthesis can be carried out under lower process temperatures (50-60°C) and results in fewer side products, thus reducing the environmental impact.

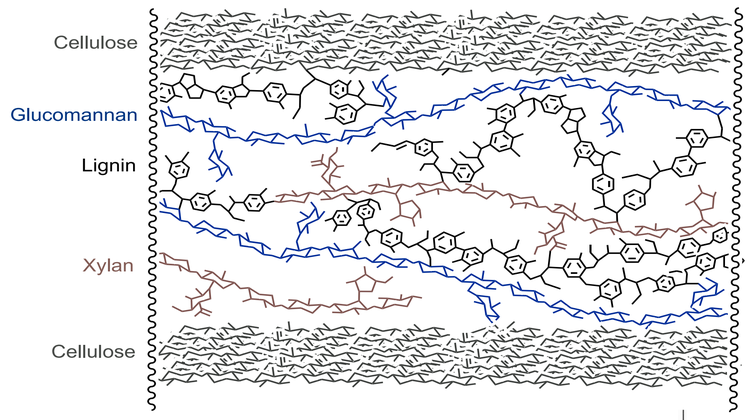


Figure 1. **Schematic representation of secondary cell wall polymers in wood** (courtesy of Gunnar Henriksson, WWSC).

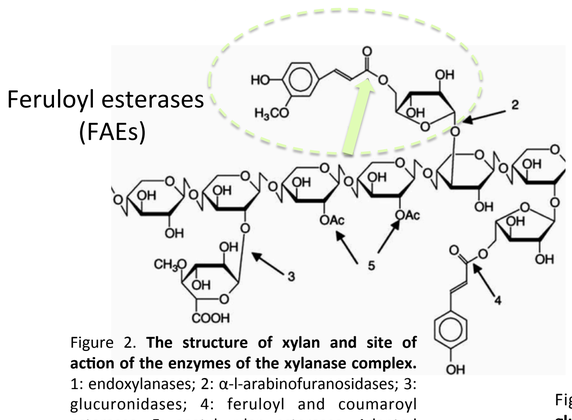


Figure 2. **The structure of xylan and site of action of the enzymes of the xylanase complex.** 1: endoxylanases; 2: α-l-arabinofuranosidases; 3: glucuronidases; 4: feruloyl and coumaroyl esterases; 5: acetyl xylan esterases. Adapted from Chávez et al., 2006 [3].

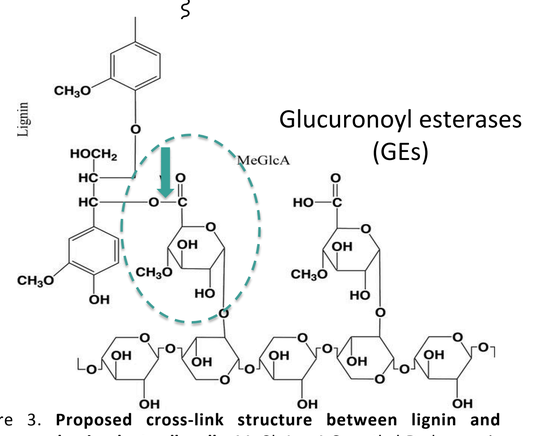
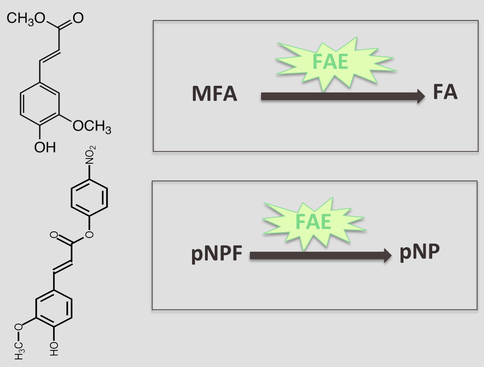


Figure 3. **Proposed cross-link structure between lignin and glucuronoxylan in plant cell walls.** MeGlcA = 4-O-methyl-D-glucuronic acid. The arrow indicates the ester bond hydrolyzed by GE. Adapted from Wood et al., 2008 [4].

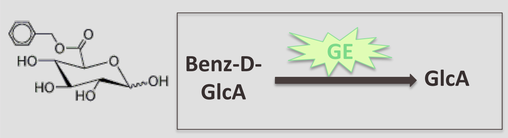
Methods & Results

Hydrolytic assays

FAEs

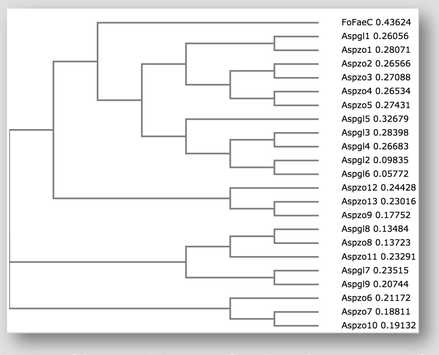


GEs



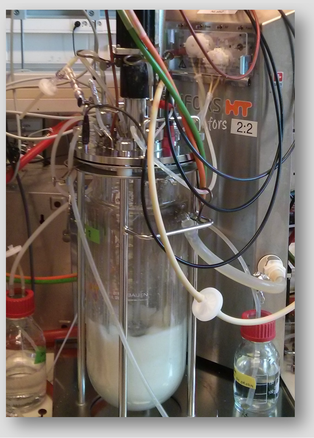
For screening of they hydrolytic activity of FAEs and GEs, spectrophotometric assays were used

Genome mining



Homologues of known FAEs were found in the genomes of two cold-tolerant Aspergillus strains (*A. glaucus* and *A. zonatus*), several candidate genes were cloned and subsequently expressed in *Pichia pastoris*.

Production of fungal enzymes in *Pichia pastoris*



Initial screening of 19 fungal strains (mesophilic and thermophilic) isolated from soil, wood and agricultural compost in Vietnam

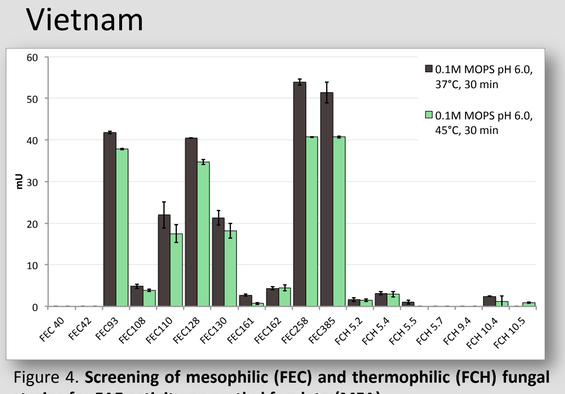


Figure 4. **Screening of mesophilic (FEC) and thermophilic (FCH) fungal strains for FAE activity on methyl ferulate (MFA).**

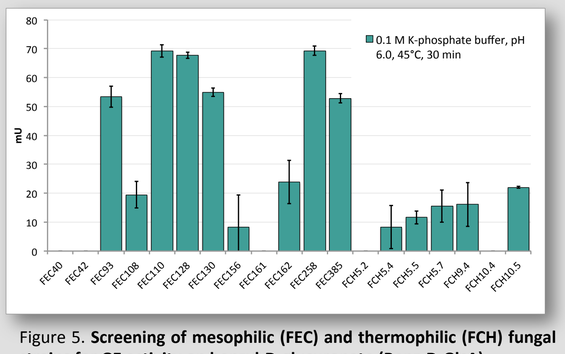
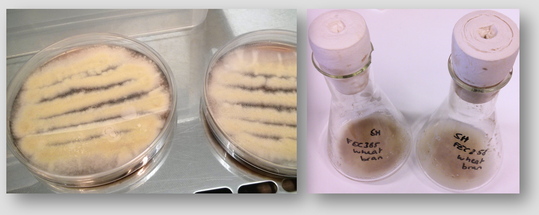


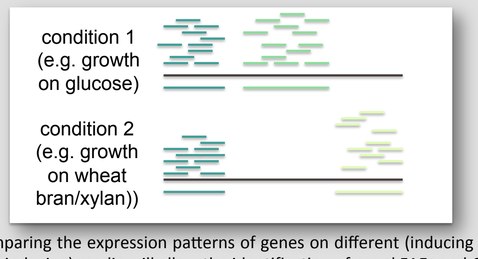
Figure 5. **Screening of mesophilic (FEC) and thermophilic (FCH) fungal strains for GE activity on benzyl-D-glucuronate (Benz-D-GlcA).**

DNA & RNA sequencing

Two thermophilic fungal strains were chosen for DNA and RNA sequencing. Mycelium was grown for 48h at 50°C on glucose, washed thoroughly and subsequently divided between shake flasks containing 1% glucose, 1% wheat bran, 1% beechwood xylan or 1% wheat bran + 0.01% ferulic acid as the sole carbon source. Samples were taken at different time points, RNA extracted and sequenced.



Selection of novel FAEs/GEs



Comparing the expression patterns of genes on different (inducing and non-inducing) media will allow the identification of novel FAEs and GEs. These enzymes will then be cloned and overexpressed in the heterologous host *Pichia pastoris*.

Transesterification reaction

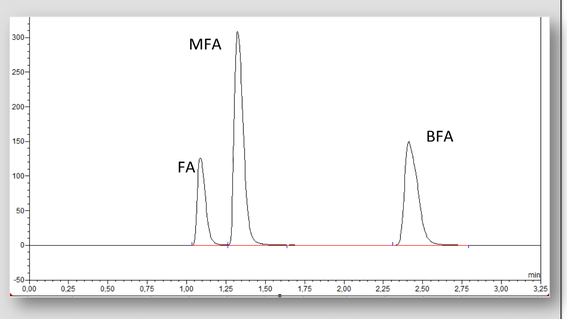


Figure 6. **Example of a chromatogram obtained from HPLC analysis of the products of the transesterification reaction methyl ferulate (MFA) to butyl ferulate (BFA) in a reaction system with 92.5% 1-butanol and 7.5% buffer.**

In a reaction system with very low water content, FAEs preferably carry out a (trans)esterification reaction.
We used a *Myceliophthora thermophila* FAE to produce butyl ferulate (BFA) from methyl ferulate (MFA) in a binary reaction system consisting of 1-butanol and 7.5% buffer, pH 6.5. At 30°C, after 69h more than 55% of MFA is converted to BFA, while only 14% of MFA is hydrolysed to ferulic acid (FA).

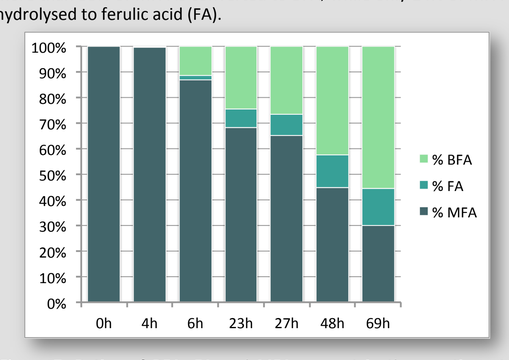
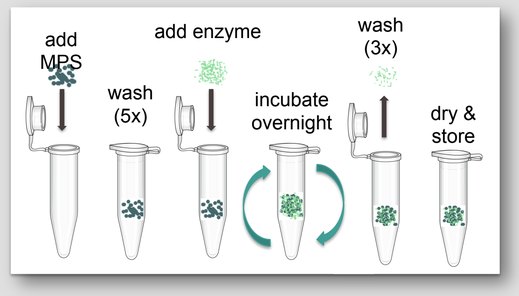


Figure 7. **Ratios of BFA, FA and MFA present in the reaction at different time points.**

Immobilisation on mesoporous silica particles (SBA-15)

Immobilisation of enzymes can improve the stability and reusability of the biocatalyst, and can also change the substrate specificity [5]. SBA-15 (Santa Barbara Amorphous material) is a well-ordered, hexagonal mesoporous silica (MPS) matrix with a controllable pore size. It is an attractive support material, as it has a very large surface area that can be functionalized if desired, and properties such as pore structure can be adjusted to individual needs.
Immobilisation on MPS is done by simple adsorption. The enzyme loading is estimated by measuring protein concentration in the supernatant after immobilisation. Adsorption is mediated mostly by electrostatic and hydrophobic interaction, thus the pH of the used buffer is very important for immobilisation efficiency.



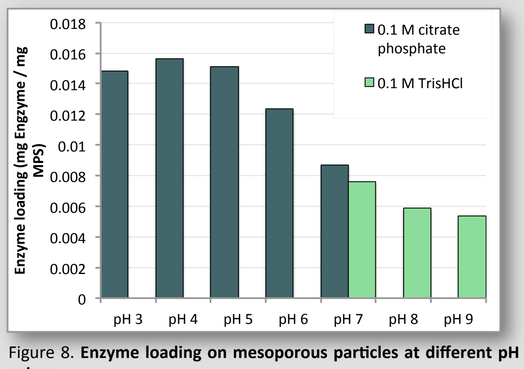


Figure 8. **Enzyme loading on mesoporous particles at different pH values.**

Conclusions

We characterised new FAE and GE enzymes from mesophilic, thermophilic and cold-tolerant filamentous fungi produced in *Pichia pastoris*. The enzymes were characterised for both their hydrolytic abilities on various model substrates (methyl ferulate, pNP-ferulate) - for potential applications in deconstruction of lignocellulosic materials and extraction of valuable compounds - as well as for their biosynthetic capacities. We tested and optimised the FAEs' transesterification capabilities on ferulate esters in a 1-butanol-buffer system, with the aim of using the most promising candidates for the production of antioxidant compounds with improved hydrophobic or hydrophilic properties, such as prenyl ferulate, prenyl caffeate, glyceryl ferulate and 5-O-(trans-feruloyl)-arabinofuranose.

Citations

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[5] Thörn, C., Gustafsson, H., & Olsson, L. (2011). Immobilization of feruloyl esterases in mesoporous materials leads to improved transesterification yield. Journal of Molecular Catalysis B: Enzymatic, 72(1-2), 57–64. doi:10.1016/j.molcatb.2011.05.002