# Esterases of basidiomycetes as supporting enzymes in degradation of lignocellulosic material

Martin Rühl[1], Alexandra Rojkova[2], Ivan Zorov[2,3], Arkady P. Sinitsyn[2,3], Holger Zorn[1]

1. Institute of Food Chemistry and Food Biotechnology, Justus Liebig University Giessen, Heinrich-Buff-Ring 52, 35392, Gießen
2. A.N.Bach Institute of Biochemistry RAS, Leninsky pr., 33-2, 119071, Moscow
3. Department of Chemistry, M.V. Lomonosov Moscow State University, 119899, Moscow

## Introduction

In contrast to fossil resources, lignocellulose as the main part of wooden plant material is available in almost unlimited amounts. Its main components - cellulose, lignin and hemicellulose – represent an important feedstock used in various industries, such as in paper manufacturing or bioethanol production. Unfortunately, lignocelluloses are recalcitrant materials, and harsh chemical conditions are needed to degrade them. In nature, fungi are capable of breaking up the wooden material by using a diverse set of extracellular enzymes, the so-called secretome. For degradation of the lignin, a number of oxidases including lignin peroxidases, manganese peroxidases, versatile peroxidases, and DyP-type peroxidases and laccases, are secreted which are able to oxidise aromatic and phenolic parts of the lignin structure. As a second part of the lignocellulosic fungal secretome, hydrolytic enzymes, such as cellulases and esterases, are needed for the extraction of sugars and are involved in the degradation of the lignocellulosic structures, respectively. The different cellulases are able to fracture the cellulose structure, whereas esterases (EC 3.1.1.x) are involved in the hydrolysis of several ester bonds. E.g., feruloyl-esterases (EC 3.1.1.73) participate in the breakup of linkages connecting hemicellulose (arabinoxylans) and lignin. Nevertheless, the knowledge on basidiomycete esterases is fragmentary and, thus, their biotechnological potential unknown.

## 1. Screening

More than 25 basidiomycetes were tested in a screening for their ability to hydrolyse three different triglycerides: Tributyrin ($C_{15}H_{26}O_6$), Tricaprylin ($C_{27}H_{50}O_6$), and Triolein ($C_{57}H_{104}O_6$).

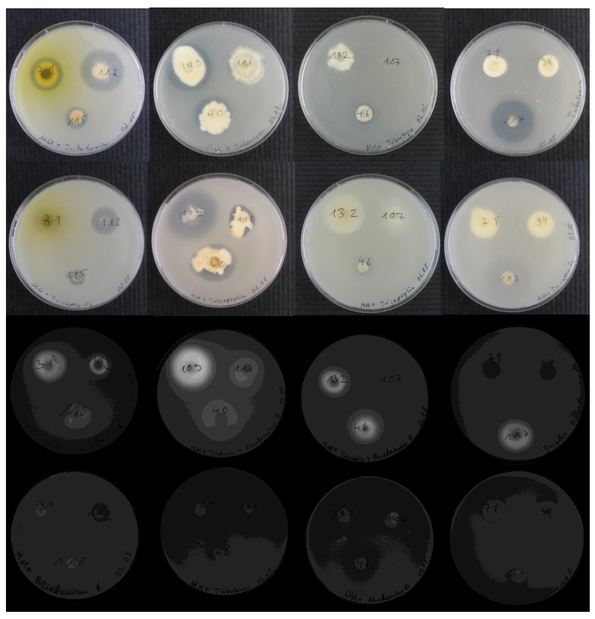


Fig. 1: Minimal medium (MM) agar plates containing aspartate, $NH_4NO_3$, $KH_2PO_4$, $MgSO_4$ and micronutrients were supplemented with the triglycerides Tributyrin, Tricaprylin and Triolein. In case of Triolein, the fluorescent dye Rhodamine B was added as described in Kouker and Jaeger 1987. Esterase/Lipase activity can be determined by clearing zones (Tributyrin and Tricaprylin) or fluorescent halos (Triolein).

| Number | Strain | Tributyrin | Tricaprylin | Triolein |
|---|---|---|---|---|
| 2 | *Trameters sp.* | ++ | ++ | - |
| 11 | *Cyathus striatus* | + | + | - |
| 24 | *Laetiporus sulphureus* | - | - | - |
| 31 | *Pycnoporus sanguineus* | + | + | ++ |
| 32 | *Stereum* sp. | ++ | (+) | - |
| 40 | *Marasmius cohortalis* | + | + | + |
| 41 | *Coprinus erythrocephalus* | + | ++ | - |
| 46 | *Trametes hirsuta* | + | + | + |
| 53 | *Agrocybe arvalis* | + | - | + |
| 59 | *Hypholoma capnoides* | - | + | - |
| 60 | Kuehneromyces mutabilis | - | - | - |
| 67 | *Fistulina hepatica* | - | - | - |
| 78 | *Coprinus clastophyllus* | (+) | - | - |
| 89 | *Coprinus sterquilinius* | + | + | - |
| 93 | *Coprinopsis cinerea* | ++ | - | - |
| 95 | *Phanerochaete chrysosporium* | - | - | - |
| 107 | *Auricularia mesenterica* | - | - | - |
| 109 | *Dichomitus squalens* | + | + | ++ |
| 111 | *Irpex consors* | - | + | + |
| 112 | *Merulius tremellosus* | ++ | ++ | ++ |
| 115 | *Pleurotus flabellatus* | + | + | + |
| 117 | *Stereum rameale* | ++ | - | + |
| 118 | *Termitomyces albuminosus* | + | + | - |
| 130 | *Heterobasidion insulare* | + | + | - |
| 132 | *Microporus affinis* | - | - | + |
| 133 | *Phanerochaete tamariciphila* | - | - | - |
| 150 | *Amylostereum areolatum* | + | + | + |

Table 1: Results of the agar plate screening ( -: no activity; (+): only faint activity; +: activity; ++: strong activity)

## 2. Esterase production

Up to know, 5 of those basidiomycetes which revealed high esterase and/or lipase activity in the agar plate screening assay were grown in liquid media (Fig. 2). In shaken flask cultures supplemented with milled poplar or pine wood, activities of up to 340 U/L were detected (Table 2). Without addition of milled wood only low activities were measured in the supernatants of fungal cultures with the complex medium (SNL): 6–25 U/L.

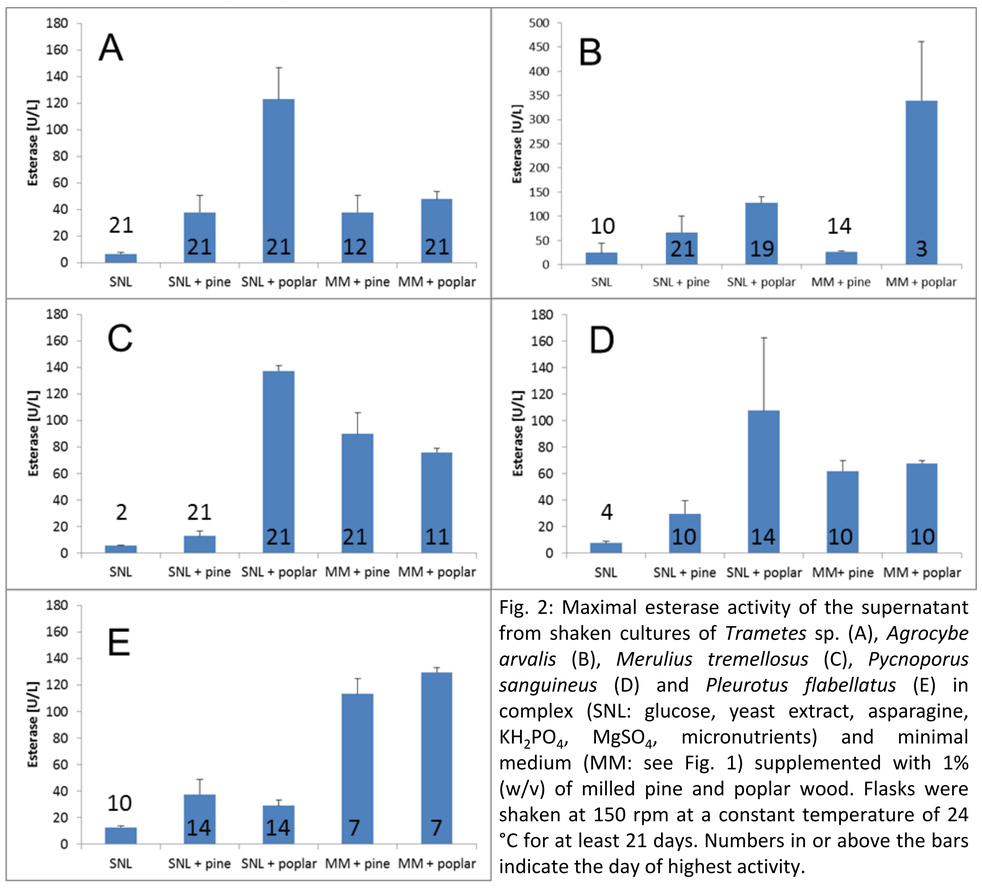


Fig. 2: Maximal esterase activity of the supernatant from shaken cultures of *Trametes* sp. (A), *Agrocybe arvalis* (B), *Merulius tremellosus* (C), *Pycnoporus sanguineus* (D) and *Pleurotus flabellatus* (E) in complex (SNL: glucose, yeast extract, asparagine, $KH_2PO_4$, $MgSO_4$, micronutrients) and minimal medium (MM: see Fig. 1) supplemented with 1% (w/v) of milled pine and poplar wood. Flasks were shaken at 150 rpm at a constant temperature of 24 °C for at least 21 days. Numbers in or above the bars indicate the day of highest activity.

| Fungus | Maximal esterase activity |
|---|---|
| *Agrocybe arvalis* | 339 ± 122 U/L in MM + poplar |
| *Trametes* sp. | 123 ± 24 U/L in SNL + poplar |
| *Merulius tremellosus* | 137 ± 4 U/L in SNL + pine |
| *Pycnoporus sanguineus* | 107 ± 55 U/L in SNL + poplar |
| *Pleurotus flabellatus* | 130 ± 4 U/L in MM + poplar |

Table 2: Maximal esterase activity of each strain in their culture supernatants.

## 3. Characterisation

To reveal the number of different esterase isoenzymes present in the supernatant, an isoelectric focusing electrophoresis (IEF) was conducted. The activity staining with an esterase substrate revealed up to 5 different bands per fungus with isoelectric points (pI) between 3.5 and 8.3 (Fig. 3). In case of *Trametes* sp. the supernatant was fractionated with an anion-exchange chromatographic column (DEAE Sepharose FF). The subsequent native SDS-PAGE (Fig. 4) with esterase activity staining showed the separation of isoenzymes into two fractions. Esterases of Fraction 1 had better ability to hydrolyse longer chain fatty acid esters than enzymes of Fraction 2. Both fractions were tested for their ability to hydrolyse different p-nitrophenyl esters (Table 3). Both fractions were not capable of hydrolysing the lipase substrate p-nitrophenyl palmitate.

As a first proof of principle, we conducetd a saccharification of milled pine wood with cellulolytic enzymes from *Penicillium verruculosum* and supernatant of *Trametes* sp. liquid cultures (Fig. 5).

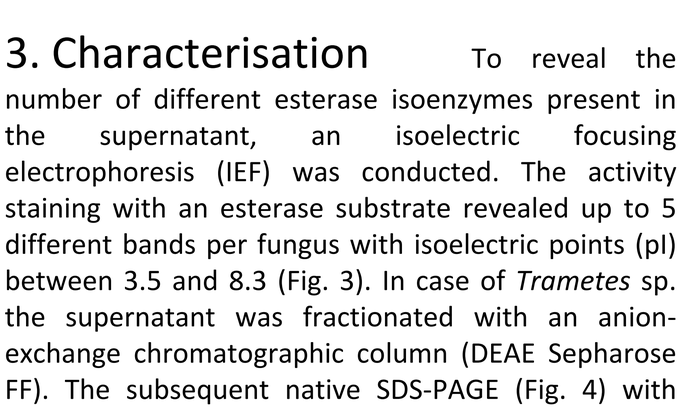


Fig. 5: Concentration of reducing sugars measured in the supernatant of the saccharification of pine wood. 5: 5 mg cellulase mix / g dry substrate, +: 40 U β-glucosidase / g dry substrate; TVI: supernatant of *Trametes* sp. liquid culture.

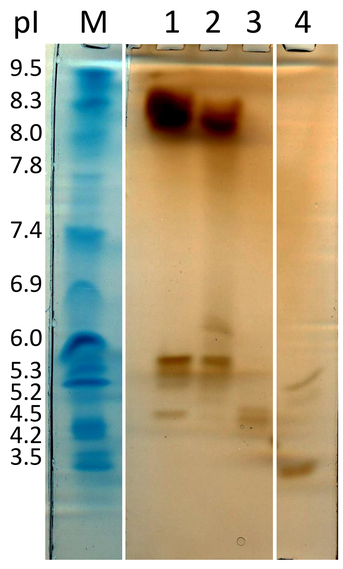


Fig. 3: Isoelectric focusing of supernatants from different fungal cultures: *A. arvalis* (1), *M. tremellosus* (2), *P. sanguineus* (3), *Trametes* sp. (4). The gel was stained with α-napthyl acetate and Fast Blue RR salt. The gel with the marker (M) was stained with Coomassie Brilliant Blue.

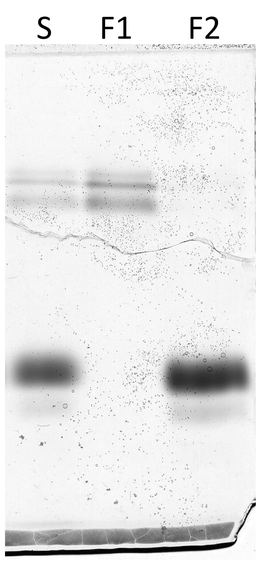


Fig. 4: Semi-native SDS-PAGE with *Trametes* sp. supernatant (S) and anion exchange chromatographic fractions F1 and F2. The gel was stained with α-napthyl acetate and Fast Blue RR salt.

| Substrate | Relative activity [%] | | |
|---|---|---|---|
| | Supernatant | Fraction 1 | Fraction 2 |
| p-nitrophenyl acetate | 100 ± 6 | 100 ± 11 | 100 ±9 |
| p-nitrophenyl butyrate | 66 ± 3 | 71 ± 3 | 13 ± 0 |
| p-nitrophenyl valerate | 49 ± 9 | 76 ± 3 | 13 ± 2 |
| p-nitrophenyl octanoate | 155 ± 9 | 187 ± 47 | 50 ± 13 |
| p-nitrophenyl palmitate | no activity detected | | |

Table 3: Relative esterase activity of *Trametes* sp. regarding hydrolysis of different esterase and lipase substrates.

## 4. Outlook

Besides the characterisation of the native esterases, we are in process to express *T. versicolor* esterases heterologously in the cellulase producing strain *Penicillium verruculosum*.




Fig. 6: Phylogenetic tree of aligned esterase protein sequences derived from sequenced genomes and/or already characterised proteins of the basidiomycetes *Coprinopsis cinerea* (Copci), *Pleurotus ostreatus* (PleosPC15), *Trametes versicolor* (Trave), *Phanerochaete chrysosporium* (Phchr) and *Schizophyllum commune* (Schco) as calculated in the program MEGA. Numbers refer to protein IDs listed at the JGI portal (http://genome.jgi-psf.org/). Already characterised esterases are encircled.

**References**: Kouker and Jaeger 1987, Applied and Environmental Microbiology 53:211-213. Spanikova and Biely 2006, FEBS Letters 580:4597–4601. Hashimoto et al. 2010, Bioscience, Biotechnology and Biochemistry 74:1722-1724. Vanden Wymelenberg et al. 2010, Applied and Environmental Microbiology 76:3599–3610.

**Acknowledgements**: We thank the Federal Ministry of Education and Research and the Foundation for Assistance to Small Innovative Enterprises for supporting the project RUS 10/B34.

JUSTUS-LIEBIG-UNIVERSITÄT GIESSEN