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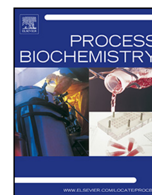

Industrial by-products from white-rot fungi production. Part I: Generation of enzyme preparations and chemical, protein biochemical and molecular biological characterization

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ABSTRACT

Industrial by-products of white-rot fungi cultivation, in particular *Lentinula edodes*, *Hericium erinaceus*, *Stropharia rugosoannulata*, *Fomes fomentarius* and *Grifola frondosa*, were screened in terms of composition and selected enzyme activities for potential biorecycling to produce economic profitable enzyme preparations. Spent growth substrate of *Lentinula edodes* cultivation was proven as natural resource rich in enzymes for conversion of lignocellulosic biomass. Subsequently, a first protocol for the recovery of lignocellulolytic enzymes was established. The chemical composition, protein profile, selected enzyme activities and hydrolyses of plant material with low digestibility were determined to study the biocatalytic potential of raw and processed by-products from *Lentinula edodes* cultivation. The analysis of proteome data showed diverse proteins for cellulose, hemicellulose and lignin conversion and revealed that glucanase was prevalent in comparison with identified proteins. Lignocellulolytic activities were measured at acidic (pH 4.5/30 °C) and neutral (pH 7.5/38 °C) conditions. The purification and stabilization of enzyme extract to enzyme preparation by ultrafiltration and salt/sugar application led to an increase in protein quantity and xylanase activity to a value of 35.72 mg/ml (+514%) and 133.01 U/ml (+299%). Polysaccharide degradation of hardly degradable hay and straw could be doubled in acceleration at pH and temperature optimum (pH 4/30 °C/24 h).

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1. Introduction

The number of mushroom producing companies, the production quantity as well as the diversity of mushrooms cultivated is rising and thus the importance of mushroom production. World mushroom production has increased since 1990 and up to 25 times over the last 35 years. In 1978 one billion kilogram were produced whereas in 2012 already 27 billion kilogram of mushrooms have been harvested. Most cultivated fungi are *Agaricus* sp., *Pleurotus* sp., *Lentinula edodes*, *Auricularia* sp. and *Flammulina velutipes*; constituting 85% of the world mushroom supply. Worldwide annual production volume amounts to approx. 4.5 million tons for *Lentinula edodes* mushroom [1]. For every kilogram of mushroom produced 5 kg spent mycelium substrate (also named spent mushroom substrate, by-products of mushroom production, spent growth substrate or waste mushroom medium) remains. The ratio of mushroom yield and raw cultivation substrate is maximum 1:5. That is why by-products of *Lentinula* cultivation have an amount of approx. 22.5 million tons. It is proving complicated to store or utilize large quantities of mushroom by-products; the disposal can become a problem for mushroom producers.

Strategies for practical utilization are e.g. composting, the usage as organic fertilizer blended into agricultural soils and soil improvement agents [2]. Valuable by-products of edible mushroom cultivation are partially discarded, although they are more advantageous than other lignocellulosic biomass, due to high density, availability throughout the year as well as biochemical modification by fungal enzymes and thereby decreased structural rigidity. It is additionally enriched with protein [3,4]. Hence, new fields of application with economical benefits are investigated. Studies for biorecycling of by-products focus on biogas production, ethanol production, enzymatic saccharification for potential generation of bioethanol, biofertilizer or bioproducts, hydrolyzate generation for

Abbreviations: WS, Wheat straw; H, Hay; CR, Chopped residue; PJ, Pressed juice; PT, Prototype; LE, *Lentinula edodes*; HE, *Hericium erinaceus*; FF, *Fomes fomentarius*; SR, *Stropharia rugosoannulata*; GF, *Grifola frondosa*; DD, *Disporotrichum dimorphosporum*; AN, *Aspergillus niger*.

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cultivation of *Lactococcus lactis*, production of plant hormones, replacement of peat moss, for cultivation of mushrooms as well as animal feed [2,3,5,6]. The review of Phan & Sabaratnam [6] shows as well solutions for utilizations, for example, by bioremediation of pollutants. This includes the biodegradation of polycyclic aromatic hydrocarbons, phenolic compounds, biocides and fungicides, petroleum, dyes and acid mine drainage as well as biosorption of heavy metals. The majority of studies were performed with by-products from *Pleurotus* sp. cultivation. The incineration and burning is neither economically practical nor ecologically friendly and for this reason no solution in waste management of spent mushroom substrate.

As stated by Phan & Sabaratnam [6] the recovery of enzymes to produce value-added products could be an option for biorecycling of the enzyme-containing by-products. The types of enzymes produced by mushrooms during cultivation are influenced by the substances of growth substrate and mushroom species. A recovery strongly depends on pH, temperature, extraction medium, incubation time, inocula density and nitrogen source.

Most investigations were carried out by the use of fruiting bodies or mycelium of fungi, not the by-products of mushroom cultivation. Ball & Jackson [7] examined the enzyme activities of diverse lignocellulolytic enzymes (xylan-, cellulose- and lignin-degrading enzymes) in blended extracts from spent mushroom compost of *Agaricus bisporus* as well as the ability of the enzyme extract to convert wheat straw. Mayolo-Deloisa et al. [8] established a protocol for the successful recovery and purification of laccase from *Agaricus bisporus* by-products. Extraction optimization and enzyme activity assays of enzymes were part of investigations on enzymes of *Pleurotus sajor-caju* by-products. The enzymes α-amylase, cellulase, β-glucosidase, laccase and xylanase have been determined by Ko et al. [9] in extracts from by-products of *Lentinula edodes*, *Pleurotus ostreatus*, *Flammulina velutipes* and *Hericium erinaceus*. It was shown that the by-products can be used as suitable source for enzyme production. Pectin-degrading enzymes were not considered even though various basidiomycetes such as *Lentinula edodes* are pectin decomposer [10]. Furthermore, our research revealed that no protocols for generation of stable enzyme preparations from by-products of white-rot fungi cultivation for industrial biotechnology purposes exist.

The aim of this study was to generate an enzyme cocktail from remained growth substrates after white-rot fungi cultivation, a renewable resource from mushroom industry, with a broad spectrum of enzymes for lignocellulose conversion in bioprocesses. Hence, chemical composition, activities of selected enzymes and the bioconversion of heavily degradable biomass were examined as a first approach, considering pectinolytic activities. It was specialized on white-rot fungi known for decomposition of complete lignocellulose and as comprehensive pool of various and unique enzymes. Beside *Lentinula edodes* and *Hericium erinaceus* by-products of *Stropharia rugosoannulata*, *Fomes fomentarius* and *Grifola frondosa* cultivation were investigated. However, spent growth substrate of *Lentinula edodes* was chosen for more intensive studies. To increase the enzyme concentration and activity of enzyme extracts from by-products, the extract was processed by centrifugation, ultrafiltration and stabilization to obtain prototypes; novel enzyme preparations. The prototypes were biochemically characterized by proteome studies to identify and quantify proteins, molecular weight determinations of proteins, enzyme activity assays and plant hydrolyses for determining the pH and temperature optima. Furthermore, the chemical composition of the enzyme preparations were analyzed.

2. Material and methods

2.1. Materials

2.1.1. Biomass

Wheat straw (WS) and hay (H) mixed 1:1 (w:w) were used as model substrate in enzymatic hydrolyses, representing a substrate difficult to digest. It was mechanically pretreated and provided by project partners of the University of Hohenheim (Germany), Unterer Lindenhof, in August 2009. Hay contained mainly ryegrass from extensive grassland. The theoretically chop length ranged between 3–5 mm.

2.1.2. By-products of mushroom production

Growth substrates for industrial cultivations of mushrooms consisted standardly of 65% water and 35% solids. The solid fraction contained 70 or 80% wood chips and 20 or 30% cereals. After mixing solids and water 1% calcium carbonate was added subsequently. A range of enzyme-containing materials made of by-products from mushroom cultivation for an application in composition analyses, proteome analyses, enzyme activity assays and enzymatic hydrolyses were provided by Pilzhof Dr. Schulz (Germany). The by-products of the following white-rot fungi were under investigation: *Lentinula edodes* (in Japanese: Shii-take), *Hericium erinaceus* (Yamabusi-take), *Stropharia rugosoannulata* (Saketsuba-take), *Grifola frondosa* (Mai-take) and *Fomes Fomentarius* (Tsurigane-take). The by-products have been recovered after mushroom cultivation and harvesting fruiting bodies (first harvest). By determining by-products of *Lentinula edodes* production from second or third harvests influences of varied harvest frequencies on the activity of selected enzymes could be analyzed.

2.1.3. Preparation of by-products

Chopping (Kompostmeister 3000, Cramer) the remained by-products led to an enzyme-containing material named chopped residue (CR). The residues were watered (18, 24 or 48 h), cold pressed (10 or 20 °C) and filtered with cotton filters to obtain an enzyme extract, the pressed juice (PJ). The press process was performed with an Obst-Beeren-Saft-Wein-Presse V20 INOX of Stabilo Werkzeugfachmarkt. Concerning the processed by-products from *Lentinula edodes* cultivation, a variety of CR and PJ were obtained from different batches of mushroom cultivation. Several batches were used to provide sufficient material and compare the material quality. Different batches are marked by numbers (CR I-III/PJ I-IX).

In addition, pressed juice was centrifuged for 5 min at 2050 × *g* (Heraeus Multifuge 3SR Plus, Thermo Scientific) and concentrated. The proteins were purified by ultrafiltration (Labscale® TFF System, Millipore). Pellicon® XL 50 cassettes (Millipore) consisted of polyethersulfone. Molecules below 10 kDa could pass the membrane. Subsequently, the retentate was stabilized by adding 30% glycerol (A) or maltodextrin (4%) and sodium benzoate (0.5%) (B), resulting in two different prototypes of enzyme preparation (PT).

2.2. Chemical composition

The analyses carried out are summarized in Table 1. Volatile fatty acids quantified were lactic acid, acetic acid, propionic acid, butyric acid, valeric acid and caproic acid. The alcohols ethanol, methanol, acetone, *n*-propanol, 1,2-propandiol, 2-butanol and 1-butanol were analyzed in selected samples. Results are presented for acids or alcohols which could be detected. In terms of phenols and phenolic acids the molecules pyrocatechol, syringalehyde, vanillin, ethylvanillin, acetovanillone (apocynin), syringol, chlorogenic acid, ferulic acid, gallic acid, caffeic acid, *p*-cumaric acid, *p*-hydroxybenzoic acid, protocatechuic acid, sinapinic acid, syringic acid and vanillic acid were measured. The content of crude fat, crude

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