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Evaluation of lignocellulolytic activities of ten fungal species able to degrade poplar wood

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ABSTRACT

The study of wood decay fungi that naturally biodegrade lignocellulosic polymers has been steadily increasing during the past two decades due to their industrial and innovative applications. In this work, we compare ten species of lignicolous macrofungi which develop fruiting bodies on poplar in relation to their capacity for growing on poplar wood chips and sawdust and of secreting cell wall degrading enzymes. All the fungi studied appeared to be able to grow well in these conditions and to secrete cellulase and hemicellulase, Mn-peroxidase and cellobiose dehydrogenase, while Li-peroxidase and laccase were produced by seven and six out of the ten species, respectively. Variability in the levels of all these enzymatic activities was assessed. Two species, never investigated before, showed the best performances as regards production of cellulolytic and hemicellulolytic activities (*Lenzites warnieri*) and Mn-peroxidase (*Perenniporia meridionalis*). The highest laccase level was detected in the well known plant pathogen *Fomes fomentarius*, and the brown-rot *Daedalea quercina* proved to be the best producer of lignin peroxidase and cellobiose dehydrogenase.

1. Introduction

In spite of its strong resistance to the attack by abiotic and biotic factors, wood can be biodegraded by some fungi, and such a natural process, fundamental for the recycling of carbon, may have great potential for industrial purposes (Fackler et al., 2007). Several white-rot fungi are increasingly regarded as possible tools for delignifying bio-pretreatments of lignocellulosic materials so as to expose plant cell wall polysaccharides and favor their subsequent hydrolytic digestion by cell wall degrading enzymes (Amirta et al., 2006). This is of particular importance in order to avoid the accumulation of agroindustrial residues in the environment (Wu et al., 1986). Moreover, the production of biofuels or other chemicals and products of industrial interest from plant biomass treated with hydrolytic enzymes is increasingly carried out. Ligninolytic and cellulolytic enzymes are regarded as very useful tools to increase innovation and competitiveness for many productions linked to biotechnologies such as food, paper/pulp, textiles etc. and their economic relevance is steadily increasing (Regalado et al., 2004; Rodríguez Couto and Toca Herrera, 2006; Mendonça Maciel et al., 2010).

Degradation of lignocellulosic biomass in nature is carried out by a mixture of hydrolytic enzymes, pectinases, cellulases and hemicellulases, and oxidative enzymes such as peroxidase and laccase, (EC 1.10.3.2, benzenediol:oxygen oxidoreductase), which act in a cooperative and synergistic mode. Generally, cellulases include endo-acting (endoglucanases (EGL), EC 3.2.1.4) and exo-acting (cellobiohydrolases (CBH), EC 3.2.1.91) enzymes, which also act in a synergistic manner (Dashtban et al., 2009). Moreover, β-glucosidases (BGL; EC 3.2.1.8) hydrolyze soluble cellobiose to glucose (Henrissat, 1991), while endoxylanases can hydrolyze β-1,4 linkages in xylan, the most abundant component of hemicelluloses, and produce oligomers which can be further hydrolyzed (Dashtban et al., 2009). Nowadays fungal enzymes involved in plant polysaccharide degradation are assigned to at least 35 glycoside hydrolase (GH) families, three carbohydrate esterase (CE) families and six polysaccharide lyase (PL) families (Coutinho et al., 2009; Battaglia et al., 2011; Van den Brink and de Vries, 2011).

Lignin biodegradation by white rot fungi especially is an oxidative process and phenol oxidases are the key enzymes (Kuhad et al., 1997; Leonowicsz et al., 1999). These enzymes, collectively

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named "ligninases", include laccases and peroxidases: lignin peroxidase (LiP; EC 1.11.1.14), manganese peroxidase (MnP; EC 1.11.1.13) and versatile peroxidase (VP; EC 1.11.1.16) which combine the catalytic properties of LiP, MnP and plant/microbic peroxidases oxidizing phenolic compounds (Martínez et al., 2005). It has been widely accepted that the oxidative ligninolytic enzymes are not able to penetrate the cell wall because of their size. Thus, it has been suggested that prior to attack by these enzymes, low molecular weight diffusible reactive oxidative compounds have to initiate changes in the lignin structure (Srebotnik et al., 1988; Tanaka et al., 1999). In fact, many white and brown rot fungi have been shown to produce H_2O_2 which enters the Fenton reaction and results in the release of reactive oxygen species such as hydroxyl radicals (Suzuki et al., 2006). Different pathways for free radical generation were found in different fungal strains; one of them involves cellobiose dehydrogenase (CDH), which is able to degrade and modify all three major components of the lignocellulosic residues (cellulose, hemicelluloses and lignin) by producing different free hydroxyl radicals (Baldrian et al., 2008).

As concerns the possible applications of these enzymes, a biological pre-treatment of wood with fungi would have the advantages of low energy demand, minimal waste production and lack of negative effects on the environment (Shi et al., 2008). On the other hand, most lignicolous fungi do not produce sufficient amounts of one or more lignocellulolytic enzymes, in particular those attacking lignin, required for an efficient and economically feasible biopretreatment (Dashtban et al., 2009). For this reason, it is important to search for new fungal strains which should be able to produce a wide range of lignocellulolytic enzymes and be particularly efficient in rapid lignin degrading, using a natural wood substrate.

The aim of this work was to compare ten species of lignicolous macrofungi, selected according to their ability to develop fruit bodies on poplar wood, in relation to their capacity to grow on poplar chips and sawdust and secrete cell wall degrading enzymes in a short time. The choice of poplar, which has a very good growth rate, was due to its extensive cultivation in the plains of North Italy, especially in South Lombardy and Piedmont. Besides being an inexpensive hardwood timber for the manufacture of paper, pallets, cheap plywood and many other items, poplar wood is also used for energy purposes: upon growth in a short rotation coppice system for two to five years, it is harvested, reduced to pellets and burned in stoves.

2. Materials and methods

All the reagents were purchased from Sigma–Aldrich.

2.1. Preparation of poplar wood chips and poplar wood sawdust

Since it has been reported that the expression of plant cell wall degrading enzymes in fungi is heavily influenced by the substrate composition, we chose as a substrate poplar wood chips and sawdust, which are readily available and a realistic choice for future wood breakdown processing All the experiments on fungal growth on wood chips and sawdust were carried out on wood from a poplar clone obtained by the tree nursery "Alasia Franco vivai", Savigliano (CN), Italy, resulting from the cross *Populus deltoides* × *Populus nigra*. Trunks (diameter: 3–5 cm) cut from two year old plants were used in the experimental work. They were oven dried at 40 °C for 24 h and then cut up by an handsaw plus a pair of secateurs so as to obtain small chips of about 1 cm length. The chemical composition of such material was determined, in terms of hemicellulose and cellulose, using the DNS method and incubating the powdered wood material with a mixture of cellulases from Novozyme® (Ghoese, 1987). The lignin content was determined using the methods described in Garcia et al. (1987).

The poplar wood sawdust was obtained by means of a vibration mill (MM301 from Retsch®) in which small wood pieces were pulverized by 2 cycles at 30 vibrations/second for 30 s.

2.2. Fungal strains and culture conditions

The mycelia used for the enzymatic screening were isolated from macrofungi collected in the last four years in various northern Italian locations (Saitta et al., 2011; Altobelli et al., 2012) and added to the Mycotheque of Pavia University (DSTA) and to the Culture Collection of Miconet (Pavia University-academic spin off).

The following Agaricomycetes were selected according to their ability to develop fruit bodies on poplar wood: *Agrocybe cylindracea* (DC.) Maire (1.2.Ac1), *Bjerkandera adusta* (Willd.) P. Karst. (1.2.Ba2), *Daedalea quercina* (L.) Pers. (1.2.Dq1), *Fomes fomentarius* (L.) Fr. (1.2.Ff1), *Ganoderma lucidum* (Curtis) P. Karst. (1.2.Gl4), *Lenzites warnieri* Mont. & Durieu (1.2.Lw1), *Perenniporia fraxinea* (Bull.) Ryvarden (1.2.Pf2), *Perenniporia meridionalis* Decock & Stalpers (1.2.Pm1), *Schizophyllum commune* Fr. (1.2.Sc1) and *Trametes versicolor* (L.) Lloyd (1.2.Tv1). All the listed species are white-rot fungi except for the brown rot *D. quercina* and, according to Liers et al. (2011), the non specific wood-rot fungus *A. cylindracea*.

Cultures were maintained at 4 °C on 2% malt extract agar (MEA) plates (Biokar Diagnostics).

2.3. Mycelium growth

Three agar plugs (10 mm per side) from each strain, grown for a week in MEA Petri dishes, were used to inoculate Erlenmayer flasks with 100 ml of liquid culture medium containing a reduced amount (0.6%) of ME. After 10 days of incubation, the mycelium of each strain was filtered, by Thermo Scientific Nalgene filters (NYL 0.1 μm), under sterile conditions and 1 g was inoculated into another Erlenmayer flask (100 ml) containing a previously sterilized mixture of 2.5 g of wood chips and 0.5 g of sawdust. In addition, 15 ml of a mineral medium with the following composition (per liter): 2.0 g $NaNO_3$, 0.50 g $MgSO_4$, 0.50 g KCl, 0.010 g $FeSO_4$, 0.10 g K_2HPO_4, were added to each flask. Each fungus was allowed to grow for 7 days before being analyzed for enzymatic activities.

In each test, the mycelia inoculated into the media containing the different substrates were incubated in a climatic chamber at $T = 25$ °C, relative humidity = 60%, without shaking.

2.4. Collection of secreted fungal enzymes

In order to collect the secreted enzymes, 15 ml of sodium acetate 0.1 M buffer (pH 4.5) were added to each fungal culture flask that was subsequently kept in a shaker at 100 rpm for 24 h. The resulting suspension was then centrifuged at 5000 rpm for 15 min in order to deposit the floating wood chips and sawdust.

2.5. Biochemical assays

Protein content of the spent medium was analyzed by the Bradford® assay. An aliquot of 20 μl was added to 1 ml of the reagent and thoroughly mixed. This solution was kept in the dark for 10 min before measuring the absorbance at 595 nm. A calibration curve was drawn using as a standard different concentrations (from 0 to 2.5 mg ml^{-1} of reagent) of a BSA (bovine serum albumin) solution.

2.6. Enzymatic assays

Activity results are expressed as U/mg (U = amount of enzyme which catalyzes one micromol of substrate per minute).