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Production of lignocellulosic enzymes during growth and fruiting of the edible fungus *Lentinus tigrinus* on wheat straw

B.E. Lechner, V.L. Papinutti *

Laboratorio de Micología Experimental, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria Pab. II (1428) Buenos Aires, Argentina

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Abstract

To investigate the growth and production of lignocellulosic enzymes from *Lentinus tigrinus*, solid state fermentation (SSF) during 110 days was performed by using wheat straw as substrate. The BE obtained was 62% and three flushes of mushrooms were harvested. *L. tigrinus* showed the capacity to degrade wheat straw, causing a 21.49% decrease in lignin content and a 53.26% decrease in cellulose. The water extractives (aromatic compounds and reducing sugars) increased to 121.44% along incubation time. All of the enzyme activities were high during colonization and ligninases declined drastically during fruit body formation. After harvesting the last flush, enzyme activities increased rapidly to highest levels, except for laccase activity. Cellulases showed the maximum of activity around 90 days post inoculation. Laccase titres reached a peak of 30 U g^{-1} at day 20 and MnP had two peaks of around 750 mU g^{-1} at days 20 and 90. Aromatic compounds from lignin degradation were not inducers of ligninases. Laccase was the most stable enzyme showing complete stability after 42 h at 40 °C. All enzymes showed acid optimum pH. The minor value was recorded for laccase and endoglucanase (pH 3.0).

Keywords: Cellulases; Ligninases; SSF; Xylanases; White-rot fungi

1. Introduction

Edible fungi are important resource of proteins and are agreeable food. During the last years, more species are being cultured in the world [1,2].

At present, only a few species of genus *Lentinus* are cultivated [2]. Sobal et al. [3] reported the culture of *Lentinus levis* (Berk. & Curtis) Singer in Mexico; Khurana et al. [4] cited nutritional values of several wild edible mushrooms included *Lentinus tigrinus* (Bull.: Fr.) Fr. It stands out to possess high quantities of fibers, few sugars and low calories and, a high quantity of the amino acids phenylalanine, trheonine and tyrosine.

These fungi degrade the lignocellulosic substrate by means of hydrolytic extracellular enzymes like cellulases, pectinases and xylanases, along with oxidases and peroxidases to degrade the lignin. Lignin is a polymer of phenylpropane units connected by different C–C and C–O–C linkages. This molecule is oxidized and degraded by a ligninase system, which is composed at least by three enzyme activities: lignin peroxidase (LiP), manganese dependent peroxidase (MnP) and laccase [5]. Cellulose is a linear polymer of glucose units, which can be hydrolyzed by the action of endoglucanases, cellobiohydrolases and β-glucosidases; these enzymes are typically induced by their substrates [6]. Hemicellulose is a heterogeneous, branched polymer. The backbone of the polymer is built of sugar monomers like xylose, in this case, the enzymes involved in its degradation are named xylanases. Similar to cellulases, the xylanases can act synergistically to achieve hydrolysis.

Our work is focused to explore new species to expanding the production and consumption by commercial growers. *L. tigrinus* has a soft flesh, strong odor and agreeable to slightly farinaceous taste that make this species suitable and attractive for consumption. The aim of this work was to characterize the production of lignocellulosic enzymes

* Corresponding author. Tel.: +54 11 4576 3300; fax: +54 11 4576 3384.
E-mail address: leandru@bg.fcen.uba.ar (V.L. Papinutti).

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during growth and fruiting of *L. tigrinus* using wheat straw as a substrate, along with the study of some of their physicochemical characteristics.

2. Materials and methods

2.1. Organism and culture conditions

L. tigrinus BAFC 197 (Cepario Micológico de la Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires) was maintained in malt-extract agar (malt extract 1.2%, glucose 1%, agar 2%) at 4 °C.

Weight losses were determined by drying the content of each bag to constant weight at 80 °C.

2.2. Substrate and fruiting conditions

Traditional methods for fruiting species of *Pleurotus* were used [2]. The substrate formulation was based on wheat straw (77%), supplemented with wheat meal (20%) and $CaCO_3$ (3%). Eight polypropylene bags were filled with 100 g (dry weight) of the formulation; humidity was adjusted in the substrate (w/w) to 74% according the initial humidity content of components. Bags were stoppered with cotton plugs held by PVC cylinders and autoclaved at 123 °C for 2 h. After cooling they were inoculated with 5% (wet weight) of spawn of *L. tigrinus* BAFC 197. Bags were incubated in the dark at 25 °C for 21 days.

After bags were completely colonized by the mycelium they were moved to the fruiting rooms for 90 days. The temperature for inducing fruit bodies was 20 ± 1 °C; photoperiod of 9 h light/15 h darkness was given; the air in the cultivation room was renewed six times per hour. Watering by spray (fog type, pressure = 2 pounds/square inch) for 5 min every 3 h was automatically provided. Bags were not removed; eight cuts/bag were made to induce fruit bodies formation. Fruit bodies were cut when mature, fresh weight was recorded for 80 days after induction. Biological efficiency (BE) was calculated as [fresh weigh of harvested mushrooms/dry matter content of the substrate] × 100. Fruiting period (F) was defined as time between the maturity of the first flush to the last one.

2.3. Sampling and preparation of crude enzyme extract

The samples, taken from the bags periodically, consisted of 3 g of substrate colonized with mycelium.

Crude extract was obtained by adding distilled water to the samples from each freshly harvested culture (5:1, w/w) stirring for 20 min, followed by filtration and centrifugation. All of the steps for crude extraction were performed at room temperature. The supernatant was stored at −20 °C until needed. For all experiments, measurements were carried out in triplicate parallel cultures. The values are reported as the mean with an experimental variation less than 10%.

2.4. Analysis of proteins reducing sugars, cellulose and lignin

Soluble proteins in the crude extract were determined by the Bradford method [7] using BSA as the standard. Reducing sugars of the crude extract were assayed by the method of Somogyi and Nelson [8] using glucose as the standard. Water extractives were determined by washing substrate with distilled water at 90 °C for 2 h, substrate dry weight were determined by vacuum filtering, the filters containing the substrate were dried at 80 °C to constant weight. Cellulose (acid soluble components) and acid insoluble lignin (Klason lignin) in the dried samples were determined by the TAPPI method [9]. Values given in the figures represent the mean from triplicate independent experiments with standard error (S.E.M.) of less than 10%.

2.5. Enzyme assays

Predominant enzymes of each group were measured. Cellulases and xylanases were incubated at 50 °C; MnP and laccase were assayed at 30 °C. Laccase was determined at 469 nm ($\varepsilon_{469} = 27.5\ mM^{-1}\ cm^{-1}$) using 5 mM 2,6 dimethoxyphenol (DMP) as substrate. Measurements were made in 0.1 M sodium acetate buffer, pH 3.6 [10]. Manganese peroxidase (MnP) was determined using phenol red as a substrate. The reaction product was measured at 610 nm ($\varepsilon_{610} = 22\ mM^{-1}\ cm^{-1}$) [11]. The reaction mixture contained 50 mM succinate buffer, pH 4.5, 0.01% phenol red. The addition of H_2O_2 (0.1 mM final concentration) initiated the reaction. Endoglucanase, endoxylanase, and PMG were determined measuring the reducing sugars, produced after hydrolysis of the substrate, by the Somogyi–Nelson method [8]. Measurements were made in 0.1 M sodium acetate buffer, pH 4.8, using the following substrates: carboxymethycellulose (CMC) 0.5% for endoglucanase, xylan from oat spelts 0.2% for endoxylanase. Enzyme activity has been expressed in International Units (U), as the amount of enzyme required to release 1 μmol of product in 1 min. In terms of productivity, the activity was defined as units per gram dry substrate ($U\ g^{-1}$). Stability assays were made by measuring the remaining activities after crude enzyme incubation for 42 h at 40 °C.

3. Results and discussion

This study characterized the growth, determined the major components degraded by *L. tigrinus* in wheat straw, identified enzymes associated with medium degradation and studied some physicochemical characteristics of such enzymes. Publications on fruit body production of *Lentinus* species are scant. More information is available regarding the cultivation of *Lentinus sajor-caju* (Fr.) Fr. This species

was successfully cultivated on various lignocellulosic materials showing different BE values [12–15].

Reducing sugars increased rapidly from day 21, later decreased until F initiation where it was practically invariable. Soluble proteins increased until last sample day (Fig. 1).

The BE obtained was 62.20 ± 16.28%. Similar values were observed in *L. sajor-caju*, which produced high yields in the same substrate reaching 70.2% [16] to 97% of BE mean [17]. Three flushes of mushrooms were harvested: 54.66% of the total produce in the first flush, 41.16% during the second and only 4.18% in the third flush. The third flush was discharged from the figures due to its poor productivity. Total dry weight loss of the substrate after 110 days of cultivation was 37.2%.

First pin-heads could be observed 14–16 days after induction conditions began.

Time curses of reducing sugars and extractable proteins during 110 days cultivation are summarized in Fig. 1. Maximum lost of reducing sugars was observed around day 40 coincident with fruiting period initiation. Reducing sugars increased rapidly from day 21, later decreased until F initiation where it was practically invariable. Soluble proteins increased until last sample day (Fig. 1).

L. tigrinus degraded wheat straw, causing a 21.49% decrease in lignin content and a 53.26% decrease in cellulose in the 110-day-old cultures. The amount of water extractives increased 121.44%.

In a previous study [18] the decrease of lignin and cellulose content of hardwood sawdust were 56 and 64%, respectively, while in the present study the dry weight loss of lignin was significantly lower. The increase of water extractives was not due to solubilization of polysaccharides to reducing sugars (Fig. 1). The crude extracts turned a dark brown in color and displayed an adsorption peak at 270–280 nm indicating the presence of aromatic compounds, which were originated from dissolution of lignin [19]. Therefore, degradation of lignin released high quantities of aromatic soluble compounds, which account for such increase.

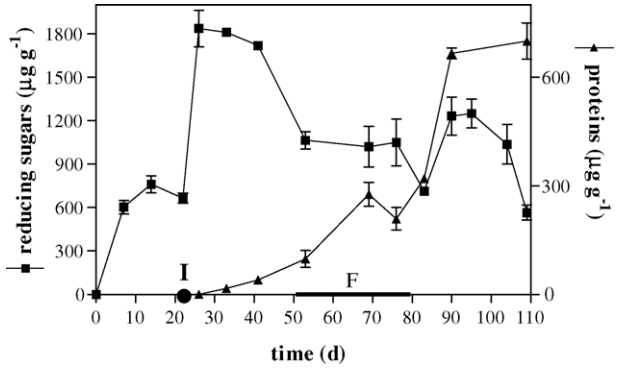

Fig. 1. Time courses of reducing sugars and soluble proteins from *L. tigrinus* growing on wheat straw. Each point represents the mean of three replicate experiments ± S.E.M. (errors not shown were within the heights of the symbols).

Table 1
Residual activity after 42 h at 40 °C and optimum pH of lignocellulases from *Lentinus tigrinus*

Enzyme	Residual activity (%)	Optimum pH
Laccase	100	3.0
MnP	65	4.5
Endoglucanase	73	3.5
β-Glucosidase	98	4.5
Endoxylanase	78	5.0
β-Xylosidase	86	5.0

Various aromatic compounds related with lignin degradation have been reported to enhance ligninases production in several white rot fungi [20–23], but the enhancement of ligninases production in response to aromatic compounds differs greatly among different white rot fungi [24,25,20]. In our experiments, extractive aromatic substances, derived from wheat straw, were not ligninase inducers. The levels of laccase and MnP production were not interconnected to total content of aromatic compounds released from wheat straw. During F, both ligninolytic activities were very low, although, accumulated aromatics compounds were higher than in the 20 first days.

Table 1 shows stability of the enzymes measured after incubation at 40 °C during 42 h. Among them, the laccase was the most stable showing complete stability after this period. All enzymes showed acid optimum pH between 3.0 and 5.0 (Table 1). The minor value was recorded for laccase and endoglucanase (pH 3.0).

Time courses of lignocellulases production are shown in Fig. 2. These enzyme activities were high during colonization, and then declined drastically or were almost constant during fruit body formation. All enzyme activities increased rapidly to highest levels after harvesting the last flush, with the exception of laccase that showed highest activity around day 20. Ligninases (laccase and MnP), these enzymes showed two maximum activities around days 20 and 90 (Fig. 2A). Laccase activity reached a peak of 30 U g^{-1} at day 20 and MnP had two peaks of 750 mU g^{-1} at days 20 and 90. Respect to cellulases, by contrast to that observed in ligninases, showed the maximum of activity around 90 days post inoculation (Fig. 2B). Both activities (endoglucanase and β-glucosidase) showed a similar pattern along the cultivation time. The increase of such activities was observed immediately after harvesting the last flush. Xylanases showed a similar profile pattern to those observed for cellulases (Fig. 2C). Maximal β-xylosidase and endoxylanase activities were observed around 90 days post inoculation.

Lignocellulosic materials posses lignin as barrier to microorganisms, this complex molecule is unable to serve them as the sole carbon and energy source [26]. White rot fungi are able to degrade lignin to reach other carbon sources present in the wood (cellulose and hemicellulose), which are a more readily utilizable source of carbon, thus the presence of a ligninase system is necessary to the degradation pro- cess, although degradation mechanisms of hydrolytic and oxidative

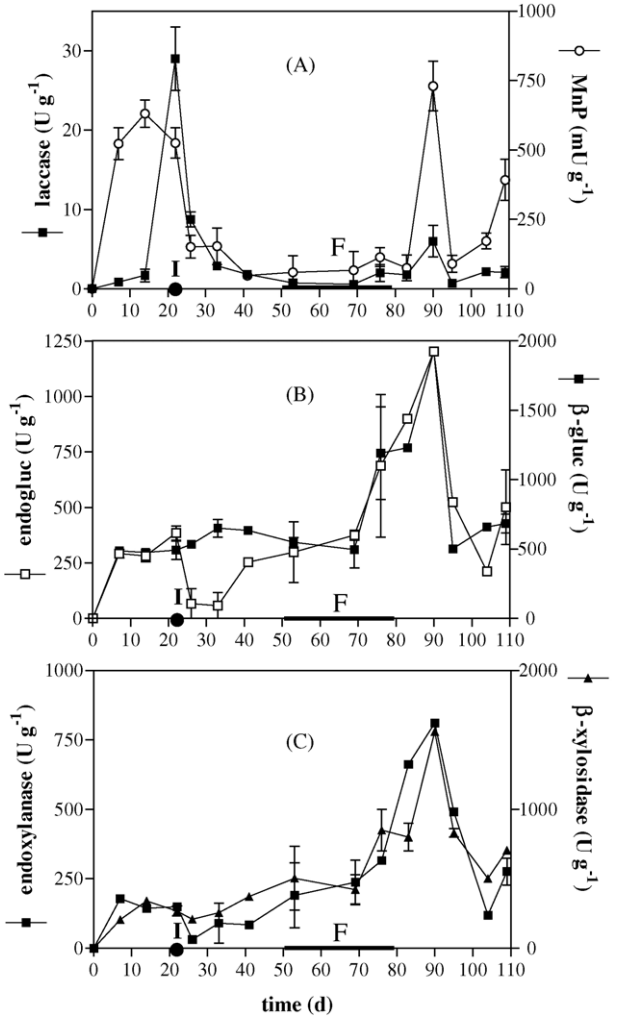

Fig. 2. Ligninase (A), cellulase (B) and xylanase (C) activities from *L. tigrinus* growing on wheat straw. Each point represents the mean of three replicate experiments ± S.E.M. (errors not shown were within the heights of the symbols).

exoenzymes are not fully understood. During trophophase or primary metabolism, the fungus produces the extracellular enzymes necessary to degrade the substrate to soluble molecules that are transported to the intracellular space and stored as glycogen; later, the glucosamine content increased to the final of incubation period, reflecting, probably, the associate preparation for fruiting [27]. The onset of fruiting is observable after a stimulus, as physiological stress, given at this moment [28]. Ligninolytic and cellulolytic activities, were present in *L. tigrinus* from day 7 and showed one or two peaks of activity before and after the fruiting period. It has been well documented that composition of the substrate influence the pattern of enzyme production by white rot fungi [29,24]. Thus, the capacity of a particular substrate to induce or increase production of lignocellulases is another factor that indirectly confers ability to grow and fruiting. In addition, not only the enzyme profiles accounts for the capacity of degradation but also their physicochemical characteristics when they are secreted; more stable enzymes at their optimum pH activity are capable to produce a more extensive degradation of the substrate. *L. tigrinus* utilized wheat straw based medium as a substrate for growth and fruiting, producing enzymes associated with its degradation such as MnP, laccase, endoglucanase, β-glucosidase, endoxilanase and β-xylosidase. All of these enzymes had high activities at pH 5.0, which was the pH recorded for substrate along 110 days cultivation time.

Previous report showed the effect of environmental stimuli on laccase and cellulase gene expression and enzyme activities in *L. edodes* cultured on supplemented sawdust [30]. Similar to observed in this work, *L. edodes* showed a rapid decrease in laccase activity at the start of fruiting. Other reports from known edible fungi such as *Agaricus bisporus* [31,32] and *L. edodes* [33] showed similar changes in laccase and cellulase activities; during development laccase activity declined before fruiting.

Several variables account for growth and fruiting on solid substrate being too complex to investigate, some of these were measured in this work. For example: enzyme activities, stability and optimum pH.

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