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ORIGINAL PAPER

Influence of the carbon source on the growth and lignocellulolytic enzyme production by *Morchella esculenta* strains

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Abstract Growth and lignocellulolytic enzymes production by two *Morchella esculenta* strains (BAFC 1728 and BEL 124) growing in solid state fermentation using different lignocellulosic materials along 58 days was characterized. Both strains were able to grow on the three substrates: wheat bran, wheat bran plus corn starch, and rolled oat. The growth was characterized by measuring chitin content, reducing sugars, pH, dry weight loss, and extractable proteins, such parameters varied substantially with substrate and strain used. The maximum rate of growth in both strains was observed between 5 and 28 days. Regarding enzyme production, as a general trend strain BAFC 1728 produced the highest titres. The most evident difference was observed in laccase production by this strain on wheat bran, which exceeded that observed in strain BEL 124 by tenfold (7.45 U g^{-1}).

Keywords Cellulases · Laccase · Ligninases · Morels · Solid-state fermentation

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Introduction

Morels are high gastronomic quality mushrooms distinguished by their textured cap, hollow body and earthy flavor. Medicinal applications have also been described, for example methanolic extracts from mycelia showed antioxidant activity and high scavenging effects on radicals [17].

The best known morel *Morchella esculenta* showed to be recalcitrant to producing fruiting bodies (ascocarps) in controlled cultures because of the necessity to develop sclerotia containing high nutrient reserve before fructification [25]. To date, there are not studies dealing with the production of extracellular enzymes associated with substrate degradation for fungal nutrition in *M. esculenta*. Fungal lignocellulases are a diverse group of extracellular enzymes able to degrade the various polysaccharides that constitute plant cell walls. Briefly, cellulose is a linear polymer of glucose units, which can be hydrolyzed by the action of endoglucanases, cellobiohydrolases and β-glucosidases. Pectin is a polymer built of a polygalacturonic acid backbone with rhamnose interruptions, with short side chains of neutral sugars, mainly arabinose and galactose. This molecule can be hydrolyzed by the action of pectinesterases, polygalacturonases, polymethylgalacturonases (PMG) and liases. Starch (amylose and amylopectin) depolymerization by amylolytic enzymes produces a mixture of dextrin, maltose, and glucose [13, 19]. Lignin is a polymer of phenylpropane units connected by diverse C–C and C–O–C linkages. This molecule is degraded by (at least) three enzymes: lignin peroxidase (LiP) [24], manganese dependent peroxidase (MnP) [10], and laccase.

To our knowledge only two articles dealing with lignocellulolytic enzymes in *Morchella* has been published. Production and characterization of cellulase complex of *M. conica* were observed in liquid cultures with crystalline

cellulose as inducer and carbon source [5]. The presence of oxidases and peroxidases was qualitatively measured in *M. conica*, *M. elata* and *M. esculenta* by spot tests in plates [12]. The objectives of this study were the characterization of growth and the production of representative lignocellulolytic enzymes by two *M. esculenta* strains growing on different solid lignocellulosic substrates.

Materials and methods

Organisms and culture conditions

Pure cultures of *M. esculenta* strains BAFC 1728 and BEL 124 were obtained from the culture collection of the Facultad de Ciencias Exactas y Naturales (Universidad de Buenos Aires). Stock cultures were maintained on malt extract (1.2%) agar (2%) slants at 4°C with periodic transfer.

The organisms were cultivated in solid-state fermentation (SSF) by using three different carbon sources: wheat bran (WB), 60% wheat bran plus 40% corn starch (WBS), and rolled oats (OAT). Humidity was adjusted in the substrate (w/w) to 75%. It was autoclaved 1 h at 121°C. After cooling media were inoculated with 5% (wet weight) of spawn grown on wheat seeds. Cylindrical flasks containing inoculated solid substrate (OAT 100 g, WB 70 g and WBS 67 g) were incubated in the dark at 20°C. Triplicate independent samples (approximately 500 mg of homogenized substrate) were collected periodically from randomly selected flasks. The growth in SSF of both strains of *M. esculenta* on different carbon sources was characterized over 58 days.

Proteins, chitin content, reducing sugars and enzyme activities

For the extraction of extracellular proteins and reducing sugars, 500 mg of solid samples from cultures were stirred 30 min at 20°C with 2.5 ml of distilled water, followed by centrifugation and filtration. Supernatant was stored at −20°C until needed for assays. Protein was determined by the Bradford method [4], with bovine serum albumin as the reference.

Entire solid cultures were dried overnight at 90°C, ground in a mortar, and stored until they were used for chitin determination. The fungal biomass content of dried solid cultures was determined by measuring *N*-acetylglucosamine (NAGA) released from chitin after hydrolysis with 6 N HCl. Analytical grade NAGA served as reference [21]. NAGA content of dry mycelia from static liquid cultures (12% malt-extract and 10% glucose) served as reference for biomass estimation of the dried solid cultures.

Reducing sugars of the crude extract were assayed by the method of Somogyi–Nelson [18] using glucose as the standard. All enzyme activities were assayed at 50°C. Laccase activity was determined by oxidation of 2,6-dimetoxyphenol (2,6-DMP) to coerulignone ($\varepsilon_{469} = 27.5\ mM^{-1}\ cm^{-1}$). The reaction mixture contained 0.1 M sodium tartrate buffer, pH 5 and 5 mM 2,6-DMP [23]. MnP activity was determined by oxidation of phenol red (0.01%). The reaction product was measured at 610 nm ($\varepsilon_{610} = 22\ mM^{-1}\ cm^{-1}$). The reaction mixture contained 0.05 M succinate buffer pH 4.8, 0.1 mM $MnSO_4$, 0.1 mM H_2O_2 [10]. LiP was determined by the H_2O_2-dependent veratraldehyde formation from 2 mM veratryl alcohol in 0.1 M sodium tartrate buffer (pH 3), reactions were started by the addition of H_2O_2 0.4 mM [24]. Endoglucanase and PMG were determined measuring the reducing sugars produced after hydrolysis of the substrate by the Somogyi-Nelson method [18]. Measurements were made in 0.1 M sodium acetate buffer, pH 4.8, using the following substrates: carboxymethylcellulose 0.5% for endoglucanase; pectin from apple 0.1% for polymethylgalacturonase (PMG). β-glucosidase activity was determined measuring the product released from hydrolysis of *p*-nitrophenyl β-D glucopyranoside (0.02%) in acetate buffer (pH 4.8). The reaction was stopped by adding Clark & Lubs buffer (pH 9.8), and absorbance was measured at 445 nm. Standard curves with glucose and *p*-nitrophenol were made to estimate enzyme activities.

Enzyme activity has been expressed in International Units (U), as the amount of enzyme needed to release 1 µg (for endoglucanase and PMG was defined as 1 µg of equivalents of glucose) of product in 1 min. In terms of production, the activity was defined as U per g dry solid substrate ($U\ g^{-1}$). All values reported are the mean of three replicates.

Results

Growth and sclerotium formation

Because of the structure and porosity of the solid substrate the mycelium penetrates and binds to it. Therefore, the fungal growth cannot be directly estimated as dry mass. Instead, growth was estimated by measuring chitin content in the dried substrate. All other variables measured contributed to the characterization of the growth of both strains in all of the three substrates. Three substrates tested supported good fungal growth (Fig. 1a, b). The greatest growth ($54\ mg\ g^{-1}$) was observed in BAFC 1728 on WB, and both strains on OAT reached biomass of approximately $45\ mg\ g^{-1}$ on the last sampling day. The most evident difference of growth between strains ($10\ mg\ g^{-1}$ and $30\ mg\ g^{-1}$ for BEL 124 and BAFC 1728, respectively) was

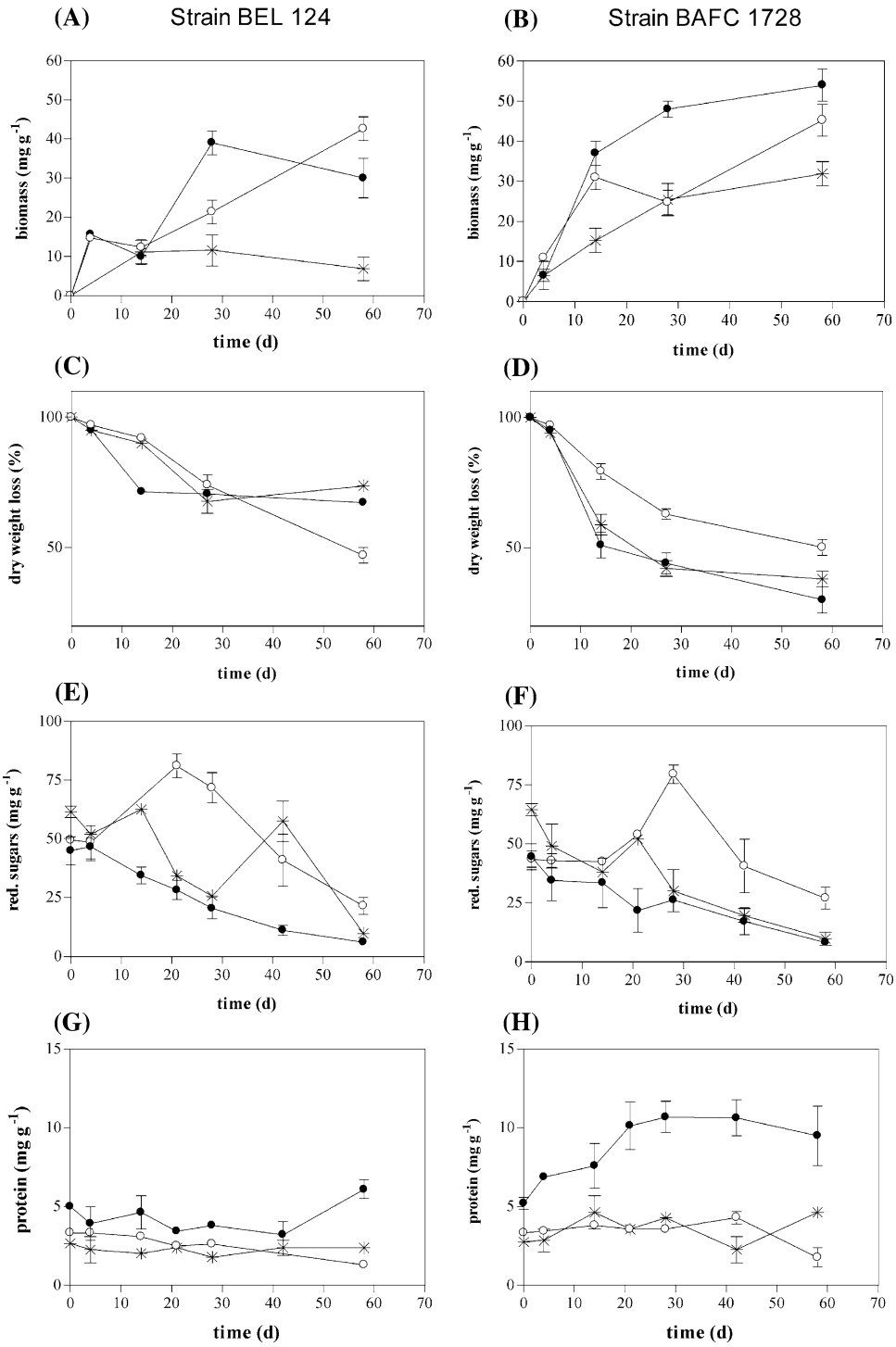

Fig. 1 Growth of two strains of *Morchella esculenta* on wheat bran (*filled circles*), wheat bran plus starch (*asterisks*) and oat (*open circles*). **a**, **b** biomass; **c**, **d** dry weight loss; **e**, **f**: reducing sugars; **g**, **h** proteins. *Error bars* denotes SEM

observed in WBS. A great difference among the mycelia propagation was also observed. While on WB and WBS the mycelium was encountered profusely immersed, in contrast, the mycelia showed a light penetration on OAT.

Regarding the dry weight loss (Fig. 1c, d), BAFC 1728 achieved the maximal value on WB (70%), then WBS (62%) and finally OAT (50%). Strain BEL 124 showed the maximal dry weight loss on OAT (53%), then on WB (35%) and finally on WBS (26%). The pH values measured along the entire cultivation period were in the range 5–6 for both strains. Reducing sugars showed different patterns when the comparison was among substrates, but were similar when made between strains (Fig. 1e, f). On OAT as substrate, the figure clearly shows a large increase of reducing sugars from 45 to 80 mg g^{-1} between days 20 and 30. From day 30 the amount of reducing sugars decreased, attaining a

lower value than the initial. On WB such increase was not observed, but reducing sugars decreased steadily from 45 mg g^{-1} to reach a minimum value of 6 mg g^{-1}. Figure 1g, h illustrates time course of extractable proteins. Strain BAFC 1728 reached the highest value on WB (11 mg g^{-1}) at day 20, after which the values remained constant. Strain BEL 124 showed similar and constant values along the cultivation time.

Conspicuous sclerotia were observed in both strains only on WBS medium at day 70. On WB the mycelium developed compact and immersed in the substrate, while on OAT it was scattered, and brown scabs were formed on the base and in contact with the glass of the flasks.

Enzyme production

The highest endoglucanase activity was reached by BEL 124 on WB with a peak value of 250 U g^{-1} at day 4 (Fig. 2a), then decreased to approximately 100 U g^{-1} and was rather constant until day 58. No peak values were observed on WBS and OAT and values were lower than on WB. The activity observed in BAFC 1728 (Fig. 2b) on WB was lower, giving peak values of 150 and 200 U g^{-1} at 4 and 42 days of cultivation, the activity on OAT was higher than BEL 124, but was reached after day 40 with no previous activity.

The highest β-glucosidase activity was observed in strain BAFC 1728 growing on WB (Fig. 2c, d). Two peak values of 1.15 and 1.65 U g^{-1} were attained at 14 and 28 days, respectively. The peak value on WB observed in strain BEL 124 was three times lower than that observed in strain BAFC 1728.

Both strains showed a quite similar time courses of PMG activity on the three media tested (Fig. 2e, f). The maximum PMG activity was measured when the strains were grown on OAT (1,500 U g^{-1}), the lowest levels were observed in the cultivations on WB (600 U g^{-1}). Amylase activity in BEL 124 on the three substrates increased until day 20 approximately and was rather constant until day 58 (Fig. 2g), being the highest activity on WBS, then WB and finally OAT, which decreased abruptly to reach lower values than WB after day 20. A different pattern was observed in the activity of BAFC 1728, being the activity on OAT higher than on WBS and WB (Fig. 2h).

Neither MnP nor LiP activities were detected in either strain under the conditions tested. The laccase activity (Fig. 2i, j) reached the maximum value on WB (7.5 U g^{-1}) in strain BAFC 1728 after 14 days, and then decreased abruptly to reach a minimum activity (0.09 U g^{-1}) at 21 days, thereafter increased to 3 U g^{-1}. A similar trend was observed in BEL 124 where two peak values were measured at 14 and 28 days showing an abrupt decrease at 21 days. Laccase activity in BEL 124 was up to ten times lower than that measured in strain BAFC 1728 being the main difference between strains respect to this activity.

Discussion

Although *M. esculenta* is a very interesting fungus from the standpoint of its taxonomy and gastronomic value, there are not any studies that focus on the growth characterization along with the production of extracellular enzymes on lignocellulosic substrates. In this work two *M. esculenta* strains were cultured in SSF by using different lignocellulosic substrates that supported a rapid and dense growth of vegetative mycelia showing similar biomass production to other typically saprobic fungi. To date, only a few reports characterized the growth of Ascomycetes in SSF. The growth in vitro of *M. elata* showed that was greatly influenced by the carbon source and pH [26]. At 58 days post inoculation, maximum biomass production for BEL 124 and BAFC 1728 was approximately 50 mg g^{-1} on OAT and WB, respectively. A similar biomass value was observed in *Giberella fujikuroi* that produced 40 mg g^{-1} according to a predicting model [9]. Hamidi-Esfahani et al. [14] studied the growth of *Aspergillus niger* (anamorph-species) in SSF and obtained amounts of 200 mg g^{-1} at 40 h. Such rapid growth of this imperfect fungus could be due to the large number of conidia (2×10^6) used as inoculum.

As a general trend, a steady decrease of reducing sugars along with high protein and biomass production on WB was observed, that was consistent with the high dry weight loss measured. Another difference observed among substrates was regarding the sclerotia formation, such structures were only observed on WBS. Previous studies on *Morchella* species indicated that the composition and the structure of the solid culture medium were main factors affecting sclerotia formation of these fungi [1]. In the present study sclerotia formation were only observed on WBS, suggesting the necessity of a substrate containing rapid metabolizable sugars, such as starch and soluble simple sugars. This beneficial effect of starch on sclerotium formation was also described by Volk and Leonard [25]. In addition, the structure of the substrate could also play an indirect role in sclerotium formation since OAT, a more compact substrate, obstructed the mycelia propagation into the solid medium, which could avoid the formation of immersed sclerotia. Amir et al. [1] demonstrated that there is a connection between sclerotium formation and translocation of nutrients from mycelium to sclerotium, which in turn is affected by the water potential gradient of the medium.

Lignocellulolytic enzyme production was extensively studied in Fungi Imperfecti, which exhibit fast growth and

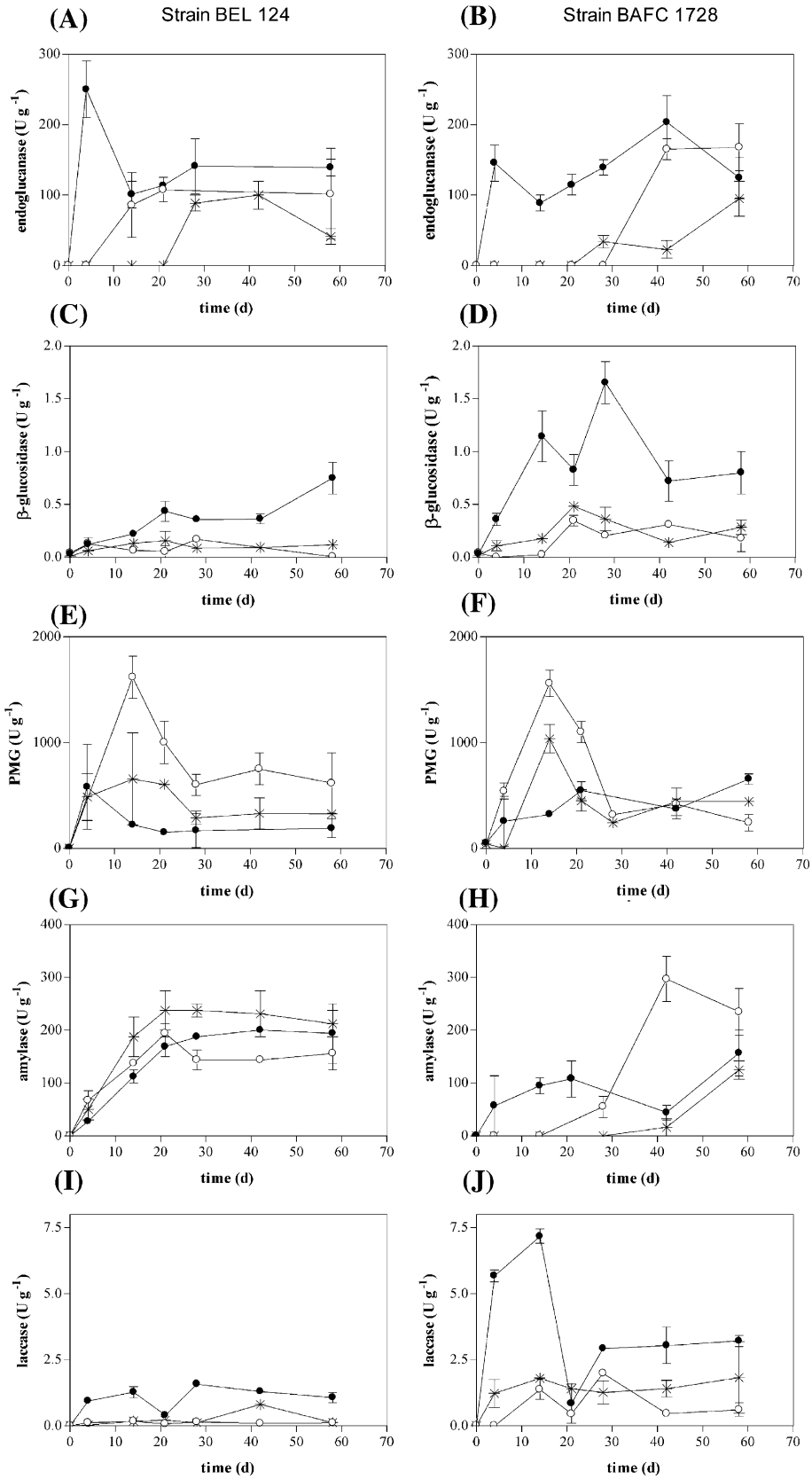

Fig. 2 Enzyme activities of two strains of *Morchella esculenta* on wheat bran (*filled circles*), wheat bran plus starch (*asterisks*) and oat (*open circles*). **a**, **b** endoglucanase; **c**, **d** *β*-glucosidase; **e**, **f** polymethylgalacturonase; **g**, **h** amylase; **i**, **j** laccase. *Error bars* denotes SEM

high production of different extracellular enzymes. In the last two decades, an intensive effort was directed to understanding the bioprocessing aspects that command enzyme production by these fungi in SSF by using a wide diversity of carbon sources. Enzyme regulation by carbohydrates was confirmed in many Ascomycetes (anamorph and teleomorph state) fungi for lignocellulolytic hydrolases, in general the corresponding substrate is the best enzyme promoter. When the production is tested in SSF such promoting effect could be overlapped by the many factors (plus their interactions) of the solid support. In this work, the production of enzymes by both strains exhibited different patterns according to the substrate and strain used. Production of endoglucanase started at the beginning in all the cultivations, but the rate and peak of production depended on the substrate used. According to other authors, cellulase production is affected by the composition of the carbon source used in the culture media, as example a higher cellulose content in the mixture resulted in higher levels of endoglucanase in cultures of *Trichoderma reesei* [19]. In fact, it was demonstrated in previous works that in many Ascomycetes, endoglucanase activity was enhanced by the corresponding substrate [16]. Similarly, in this work the highest endoglucanase levels were reached in the cultivation on WB, such substrate contains the highest percentage of cellulose compared to WBS and OAT [2]; it could also explain the differential pattern observed in PMG and amylase activities. Titres of PMG obtained in both strains of *M. esculenta* were lower than those observed in other Ascomycetes. A recent work of Botella et al. [3] reported in *A. awamori* showed maximum of approximately 7,200 U g^{-1} after 48 h growing in SSF, while the maximum reached by *M. esculenta* after 14 days of fermentation was 1,800 U g^{-1}.

It is well known that lignin is degraded by at least three ligninolytic enzymes LiP, MnP and laccase. The white rot fungi *Pycnoporus cinnabarinus* and *P. sanguineus* produce sole laccase as lignin-degrading enzyme [8, 22], suggesting that *M. esculenta* could perform lignin degradation by using only laccase as oxidative enzyme. The ligninolytic capacity was shallowly showed in *Xylaria* [15] and *Botryosphaeria* [7]. The highest laccase titre (7.5 U g^{-1}), obtained in BAFC 1728, was much lower than those reported in white rot fungi growing in SSF [6, 11, 20]; to our knowledge, there is no data about laccase production by Ascomycetes growing in solid media.

In conclusion, the resulting enzyme and growth profiles of *M. esculenta* found in the present study may help to understand the physiology of this species in SSF with different carbon sources. Both strains were able to growth on the substrates tested, forming sclerotia only on WBS. Little is know about the lignocellulolytic enzymes from *M. esculenta*, but this study showed activities of endoglucanase (50–250 U g^{-1}), β-glucosidase (100–1,700 mU g^{-1}), amylase (50–250 U g^{-1}), laccase (1–7.5 U g^{-1}) and PMG (100–1,700 U g^{-1}). It was found that higher enzyme activities generally resulted when the substrate was WB.

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