PRODUCTION OF *PLEUROTUS´*S LIGNINOLITYC ENZYMES ON COFFEE PULP BY SOLID STATE FERMENTATION

NORA GARCÍA ODUARDO[1], ROSA BERMÚDEZ SAVÓN[1], ISABELLE GAIME PERRAUD[2], SUYÉN RODRÍGUEZ PÉREZ[1], ISABEL AGUILERA RODRIGUEZ[1], HUMBERTO MORRIS QUEVEDO[1]*.

[1] Center for Studies on Industrial Biotechnology (CEBI), Universidad de Oriente, Reparto Jiménez, Santiago de Cuba 5. CP 90 500, Cuba. [2] IRD-Biotrans Unit, IMEP Case 441. Faculty of Science of Saint Jérôme, University Paul Cézanne. Av. Escadrille, Normandie-Niemen, 13397 Marseille cedex 20, France.
hmorris@cebi.uo.edu.cu

ABSTRACT

At present, there is a significant interest in solid-state fermentation (SSF) techniques as an efficient biotechnological process for *Pleurotus* cultivation and the production of a wide variety of enzymes. In this research, the production of ligninolityc enzymes by SSF, using six strains of *Pleurotus* spp. (five *Pleurotus ostreatus* and one *Pleurotus pulmonarius*) was studied. The production of laccases enzymes was evaluated during both colonization (Raimbault columns) and fructification (plastic bags) phases of mushroom growth. The influence of mixtures (1:1) of coffee pulp with cedar chip, coconut and cocoa shells in the laccase production was also investigated. *Pleurotus ostreatus* strain CCEBI 3023 produced the highest laccase activity (25 Ug^{-1}) at 96 h of growth on coffee pulp, during the colonization phase in small Raimbault column. Coffee pulp showed a maximum reduction in phenolic and caffeine contents during the first two days of incubation. Laccases appear to be involved in the detoxification of the substrate. When growing on coffee pulp in plastic bags, *P. ostreatus* CCEBI 3023 showed the greatest levels of laccase activity after fructification/harvesting (1,74 U g^{-1}) with respect to the mixtures of coffee pulp and other by-products. As judged by its complex chemical composition, the coffee pulp could act as an inducer of laccase activity. The results were compared with previous studies performed in *Pleurotus* spp. by SSF as well as submerged fermentation.

Keywords: *Pleurotus* spp., ligninolityc enzymes, coffee pulp, solid-state fermentation

INTRODUCTION

Pleurotus ostreatus is the third most important cultivated mushroom for food purpose and also it is a well studied white-rot fungus. The genus *Pleurotus* is related to the bioconversion of agricultural wastes into valuable food products and the use of their ligninolytic enzymes (laccase, manganese peroxidase, etc.) for the biodegradation of organopollutants, xenobiotics and industrial contaminants [1]. Laccase (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) catalyzes the oxidation of ortho- and para-diphenols, aminophenols, polyphenols, polyamines, lignins, and aryl diamines as well as some inorganic ions [1, 2]. Due to the broad variety of reactions catalyzed by laccases, this enzyme holds great promise for many potential industrial applications in biotechnology: bioremediation, green organic synthesis including conductive polymers, and even design of laccase-based fungicidal and bactericidal preparations [3, 4]. There is a significant interest in using solid-state fermentation (SSF) techniques to produce a wide variety of enzymes [5]. However, there is lack of information about the use of basidiomycetes in SSF using glass columns.

Coffee pulp, one of the principal byproducts of wet processed coffee (*Coffea arabica* L.), is rich in carbohydrates, proteins, minerals, and potassium. However, the presence of caffeine, tannins and polyphenols limits its utilization [6]. Nevertheless, SSF of this waste offers an

attractive recycling alternative [7]. Coffee pulp has been also used experimentally in the cultivation of mushrooms, since it represents a direct conversion of an agricultural waste to human food. Among edible mushrooms evaluated for this commercial activity, *Pleurotus* strains appear promising [8-10].

Degradation of lignocellulosic substrates by mushrooms of the genus *Pleurotus* depends on the production and secretion of enzymes such as cellulases, hemicellulases and ligninases. The production of these enzymes is important in substrate colonization and decisive in fruiting bodies production [3]. Although the increase in laccase production by *Pleurotus* mycelia is a well-studied phenomenon, there is not yet enough information on the origin of the overproduction of this enzyme.

This research was aimed to study the capacity of white-rot fungi *Pleurotus* spp. to produce laccase enzyme by SSF during both colonization (Raimbault columns) and fructification (plastic bags) phases of mushroom growth. The lost of toxic components, particularly total phenols and caffeine, was evaluated. The influence of mixtures (1:1) of coffee pulp with cedar chip, coconut and cocoa shells in the laccase production was also investigated.

MATERIALS AND METHODS

Strains. Six strains of *Pleurotus* spp. were studied: *P. ostreatus* CCEBI 3021, CCEBI 3023, CCEBI 3024 y CCEBI 3073 and *P. pulmonarius* CCEBI 3027 were obtained from the Culture Collection of CEBI (Center for Studies on Industrial Biotechnology, University of Oriente, Cuba) and *P. ostreatus* MC 50 from Culture Collection of COLPOS (Campus Puebla, México). All strains were maintained and subcultured in potato-dextrose-agar (PDA, Sigma, U.S.A.) and incubated at 25°C. Two strains (CCEBI 3023 and 3024) were selected for the study of the reduction of toxic components at different column fermenter´s size.

Substrates. Coffee pulp from *Coffea arabica* L. was collected at a coffee processing plant (El Ramón, Santiago de Cuba, Cuba), sun-dried to a moisture content of 10-15%, and then stored at environmental temperature. It was sieved to obtain a particle size of 0.8-2 mm. Coconut shells (*Cocos nucifera,* L.) from "El Cristo", were also sun dried and crushed in irregular fractions bigger than 3 cm. Cocoa shells (*Theobroma cacao* L.) from the farm "La Mandarina" (III Frente), once collected in cacao orchard were sun dried, crushed and screened to select the fraction with a particle size between 1.25 and 4 mm. The cedar chip (*Teona ciliata* Roem) was obtained from the University of Oriente's carpentry.

All the substrates were pasteurized by immersion in hot water at 70-80°C for 60 min. A solution of Benomyl (Bayer, Germany) at 0.02 % was used in order to decrease microbial contamination. In addition to pure coffee pulp, binary formulations (1:1, w/w) were prepared by mixing up the coffee pulp with other by-products (coconut shells, cocoa shells and cedar chip). For fructification studies, the pasteurized substrates were homogeneously mixed with the spawn (5%, w/w) and placed in transparent plastic bags of 2 kg (30x40 cm) [9].

Column fermenters. Colonization studies were carried out in small column fermenters (20 cm large and 25 cm of diameter) with a capacity of 30 g. The reduction of toxic components was also evaluated at medium size column (150 g) and higher size column (750 g). In these experiments the coffee pulp was sterilized 20 min at 121°C and SSF was performed according to Raimbault and Alazard [11].

Enzyme assays. Determinations in colonization studies on Raimbault columns, were made daily during seven days (a duplicate column for each fermentation time). In the case of studies made

with the substrate remnant of plastic bags after fructification/harvesting, the analysis were performed at 60 days of cultivation.

The fermented coffee pulp was extracted with sodium phosphate buffer 0.1 M pH 6.0 (50 mL of buffer per 3 g of remnant substrate) with gentle agitation in an ice bath for 30 min. After that, the suspension was filtered and then centrifuged 10 min at 4 000 rpm. Laccase activity was determined using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate [12]. Enzyme activity was expressed as international units (U), defined as the amount of enzyme required to produced 1 µmol product/min/g (dry weight).

Variation in phenolic content and caffeine in different Raimbault column's size. Phenols present in the substrate were determined by Folin and Ciocalteu reagent [13]. The analysis of caffeine was made by the Kreiser and Martin's method [14]. Samples were extracted in boiling water and filtered prior to injection on to the HPLC column. Results were expressed per gram of dry matter.

Statistical analysis: Data are expressed as means ± S.D. One-way analysis of variance were performed and where significant F values were obtained at the 5% level, differences between individual means were tested using the Duncan test.

RESULTS AND DISCUSSION

Laccase activity was measured daily during seven days of fermentation on coffee pulp of all the *Pleurotus* spp. strains in the small Raimbault columns (Fig. 1). All the *Pleurotus* strains studied grew well in these conditions. At initial growth stages, a lag phase with almost no laccase activity was detected. Subsequently, a rapid increase in the enzyme production was observed until a maximum of activity, which decreased at the end of the fermentation period. A similar pattern of laccase production was found in all the strains on small fermenter [15] in agreement with other reports [16-18]. It is noteworthy that *P. ostreatus* CCEBI 3023 showed the highest laccase activity (25 Ug^{-1}) at 96 h of fermentation, surpassing our reference productive strain CCEBI 3024 (17,9 Ug^{-1}).

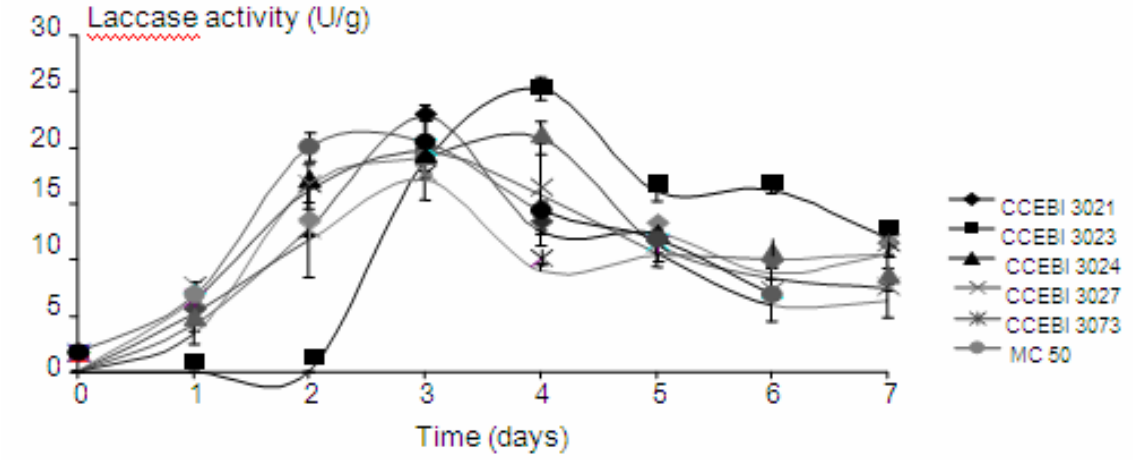

Figure 1: Laccase activity during the cultivation of six *Pleurotus* spp. strains on coffee pulp in small Raimbault columns.

Taking into account the highest laccase activity of *P. ostreatus* CCEBI 3023 found in this communication and the fact that in previous studies *P. ostreatus* CCEBI 3024 was able of decolourizing wastewaters with a high polyphenol content, such as those from pasteurized coffee pulp (laccase activity of 8.53 ± 0.73 UmL^{-1} at day 9 of cultivation) [4], these strains were chosen to assess the reduction of toxic components contained in coffee pulp by SSF at different Raimbault columns size.

Variation in the phenolic content of substrates during the culture cycle is an important aspect in the adaptation of the strain, especially since the capacity of a substrate to resist degradation has been partially attributed to its array of phenolic compounds [4, 16].

In the present study, the coffee pulp showed a reduction in phenolic content during the first two days of incubation. The 53.07% of total phenols were transformed at 24 h by the strain CCEBI 3023, and the 45.51%, at 48 h by the strain 3024. This is particularly important because a rapid reduction in phenolic concentration would accelerate colonization, and consequently, decrease the risks of invasion by mold contaminants.

The changes in enzymatic activity and phenol concentration observed during vegetative development suggest that laccase might be associated with the detoxification of soluble phenolic compounds and lignin degradation.

The caffeine content of coffee pulp also decreased during mycelial growth (Table 1). Caffeine loss was higher at day seven (last day). The results range between 15-24% and were similar to those reported by Salmones et al. [18].

The ability to produce laccase enzymes in relation to the loss of total phenols and caffeine by *Pleurotus* strains was demonstrated. This enzyme could be responsible of the transformation of these toxic compounds [12, 16, 18]. Other enzymes like peroxidases were secreted in minor activities [19]. The complex chemical composition (tannins, lignin, etc.) of coffee pulp could act as an inducer of laccase activity due to the possible enhancement of laccase gene expression.

In view of the significant laccase activity of *P. ostreatus* CCEBI 3023 and its important contribution to the overall ligninolytic enzyme activity, we also examined the extracellular laccase production of this strain cultured on coffee pulp and mixtures (1:1) in plastic bags after fructification and harvesting (60 days).

The results evidenced that coffee pulp favored the production of laccases, being the activity higher on pure coffee pulp than in the mixtures with other by-products (Table 2).

In the case of laccase activity of CCEBI 3024 strain, cultured in transparent plastic bags, a maximum of 2,41 Ug^{-1} was achieved at day 14 of cultivation [20]. The production of laccase enzyme after fructification/harvesting of *Pleurotus* spp. on mixtures of lignocellulosic substrates by SSF is reported for the first time. The highest value found in coffee pulp reflected the influence of the nitrogen content of substrate in laccase production; on the other hand, the mixture with cedar chip had the lowest nitrogen content [21]. Previous studies showed that the production of ligninolityc enzymes is closely related to the nitrogen content of substrates.

Table 1: Caffeine concentration, initial-final values (mg/g) and loss percent (in parentheses) in the coffee pulp during SSF by *P. ostreatus* strains CCEBI 3023 y CCEBI 3024.

	Fermenter type		
Strain	Small	Medium	Large
CCEBI 3023	0.52-0.40 (23.4)	0.58-0.48 (16.6)	0.54-0.41 (22.9)
CCEBI 3024	0.64-0.53 (18.5)	0.56-0.43 (24.0)	0.51-0.44 (15.2)

Table 2: Production of laccase enzyme by *Pleurotus ostreatus* CCEBI 3023 on coffee pulp and mixtures (1:1) at 60 days of cultivation.

Substrates	Laccase Activity (Ug^{-1})
Coffee pulp	$1,745 \pm 0,013^{d}$
Coffee pulp : cocoa shells	$1,234 \pm 0,004^{c}$
Coffee pulp : coconut shells	$0,447 \pm 0,016^{b}$
Coffee pulp : cedar chip	$0,308 \pm 0,036^{a}$

Average of three value ± standard deviation. Distinct letters mean significant differences (ANOVA and Duncan¨s test, $p<0.05$).

The differences between coffee pulp packed in the bags and in Raimbault column lie in the method employed in its treatment. The former is pasteurized (immersion in hot water during one hour) on which losses many soluble components, whereas the later is sterilized (at 121°C during 20 min) and components of the coffee pulp are at greater concentrations.

CONCLUSION

P. ostreatus strain CCEBI 3023 produced the highest laccase activity (25 Ug^{-1}) at 96 h of growth on coffee pulp, during the colonization phase in small Raimbault column. The maximum reduction in phenolic and caffeine contents of coffee pulp was observed in the first two days of cultivation. When growing on coffee pulp in plastic bags, *P. ostreatus* CCEBI 3023 showed the greatest levels of laccase activity after fructification/harvesting (1,74 U g^{-1}) with respect to the mixtures of coffee pulp and other by-products. As judged by its complex chemical composition, the coffee pulp could act as an inducer of laccase activity.

ACKNOWLEDGEMENTS

This work was partially supported by grants from the IRD-Biotrans Unit, IMEP, Faculty of Sciences of Saint Jérôme, University Paul Cézanne, Marseille, France.

REFERENCES

[1] Cohen R. et al. (2002). Biotechnological applications and potential of wood-degrading mushrooms of the genus *Pleurotus*. *Appl. Microbiol. Biotechnol.* 58: 582-594.

[2] Ikehata K. et al. (2004). Recent developments in the production of extracellular fungal peroxidase and laccases for waste treatment. *J. Environ. Eng. Sci.* 3: 1-19.

[3] Leonowicz A. et al. (2001). Fungal laccase: properties and activity on lignin. *J. Basic Microb*. 41:185-227.

[4] Rodríguez S. et al. (2008). Decolourisation of mushroom farm wastewater by *Pleurotus ostreatus*. *Biodegradation* 19:519-526.

[5] Viniegra-González G. et al. (2003). Advantages of fungal enzyme production in solid state over liquid fermentation systems. *Biochem. Eng. J.* 13:157–167.

[6] Bressani R. (1979). Factores antifisiológicos de la pulpa de café. In: *Pulpa de café: composición, tecnología y utilización.* Braham J.B. and Bressani R. Eds. pp 143-152.

[7] Perraud-Gaime I. et al. (2000). Adding value to coffee solid byproducts through biotechnology. In: *Coffee Biotechnology and Quality.* Sera T et al. (Eds) pp 437-446.

[8] Martínez-Carrera D. et al. (2000). Commercial production and marketing of edible mushrooms cultivated on coffee pulp in Mexico. In*: Coffee Biotechonology and Quality* .Sera T Eds. pp 471-488.

[9]Bermúdez R.C. et al. (2001). Cultivation of *Pleurotus* on agricultural substrates in Cuba. *Micol. Apl. Int.* 13(1):25-29.
[10] García N. et al. (2006). Cultivo de cepas de *Pleurotus* spp. sobre pulpa de café. *Rev Mex Micol*. 23: 99-101.
[12] Palmieri G. et al. (1997). A novel white laccase from *Pleurotus ostreatus*. *J. Biol. Chem.* 272: 31301-31307.
[13] Barlocher F. and Graca M.S.A. (2005). Total phenolic. In: *Methods to study litter decomposition: A practical guide*. Graca M.S.A. et al. Eds. pp 97-100.
[14] Kreiser W.R. and Martin R.A. (1978). High pressure liquid chromatographic determination of theobromine and caffeine in cocoa and chocolate products. *JAOAC* 61(6): 1424-1427.
[15] García N. et al. (2007). Producción de lacasa extracelular, remoción de fenoles y cafeína por *Pleurotus spp*. cultivado en pulpa de café. *Rev. Tecnol. Quím.* XXVII (3): 83-91.
[16] Mata G. et al. (2005). Changes in lignocellulolytic enzyme activites in six *Pleurotus* spp. strains cultivated on coffee pulp in confrontation with *Trichoderma* spp. *World J. Microbiol. Biotechnol*. 21: 143–150.
[17] Salmones D. and Mata G. (2002). Detection of extracellular enzymes produced by *Pleurotus* spp. grown on coffee pulp. In: *Proceedings of the Fourth International Conference on Mushroom Biology and Mushroom Products*. Sánchez et al. Eds. pp 213-219.
[18] Salmones D. et al. (2005). Comparative culturing of *Pleurotus* spp. on coffee pulp and wheat straw: biomass production and substrate biodegradation. *Biores. Technol*. 96: 537-544.
[19] García N. et al. (2010). Potencial biotecnológico de la pulpa de café para producir enzimas ligninolíticas por FES. *Rev Tecnol. Quim.* XXX(2):102-107
[20] García N. (2008). Producción de setas comestibles y enzimas lacasas por fermentación en estado sólido de la pulpa de café con *Pleurotus* spp. *Tesis de Doctor en Ciencias Técnicas.* Centro de Estudios de Biotecnología Industrial. Universidad de Oriente. 150pp.
[21] Kourouma A. (2008). Obtención de lacasas de *Pleurotus* sp. en residuales del café. *19 Conferencia de Química*. 16-18 de Dic., Universidad de Oriente. Santiago de Cuba, Cuba