

GROWTH OF *PLEUROTUS OSTREATUS* ON THE WASTEWATER OF A MUSHROOM FARM

S. Rodríguez, M. Fernández, R. C. Bermúdez, H. Morris and N. García
Centro de Estudios de Biotecnología Industrial, Facultad de Ciencias Naturales, Universidad de
Oriente. Santiago de Cuba 90500, Cuba. <suyen@cebi.uo.edu.cu>

ABSTRACT

Cultivation on solid substrate is the usual method for preparing mushroom inoculum, but liquid media offer some advantages. The production of edible mushrooms on coffee pulp substrate generates a very intense black liquid during pulp pasteurization which requires treatment before being released to the environment. In this study, the growth of *P. ostreatus* on the wastewater of coffee pulp extract (CPW) was evaluated for the possibility of obtaining biomass to be used as inoculum, and for reduction of the colour of the wastewater. The effect of the residue on growth and in biomass production was determined, and factors such as the carbon source and its concentration; the addition of certain ingredients; and decolourisation were evaluated. It was observed that *P. ostreatus* could be cultivated employing CPW supplemented with glucose. The highest yield was obtained when glucose (10 g/l) was added. It did not require the addition of supplements or salts to the synthetic medium. A typical growth in the form of homogeneous pellets was in evidence and the presence of laccase activity was detected, with values similar to those obtained in other studies. The reduction of the color of the wastewater was possible due to colored compounds that are typical substrate for laccase (e.g. condensed polyphenols). However, the polluting load (COD) diminished more than 50 % at the end of 20 days of fermentation. Color removal therefore depends on the characteristics of the wastewater treated, although other mechanisms besides laccases seem to participate in this process.

INTRODUCTION

The Center for Studies of Industrial Biotechnology has investigated the residue from mushroom farms for possible uses such as: animal food, organic fertilizer, vitamins, pectic enzymes, etc. (Bermúdez *et al.* 2001). The objective has been to obtain a clean technology with a minimal impact on the environment. Anaerobic degradation of the extract of coffee pulp, which is the main liquid residue generated, has been studied. Coffee pulp wastewater (CPW) obtained during the pasteurization of coffee pulp has a distinct color. Bermúdez and Pérez (1998) obtained removal values for its organic load of around 62% for anaerobic treatment, but no colour removal was reported. The colour of the wastewater is due to compounds with high molecular weights, such as polyphenols, condensed tannins, and caffeine.

The employment of white-rot mushrooms is an alternative for the treatment of this residue, because these organisms possess a multi-enzymatic system that allows the degradation of products with dissimilar complex structures. *Pleurotus ostreatus*, which is cultivated on our mushroom farm, is one of the four white-rot mushrooms most studied and most commercialized throughout the world. It has been employed in bioremediation to diminish the content of polycyclic aromatic hydrocarbons (Eggen 1998), in biopulping processes within the paper and pulp industry (Feijoo and Lema 1995) and in the treatment of chemical industry wastewater (Knapp and Newby 1999). It has also been used in pre-treatments to improve the digestibility of lignocellulosic wastes used in the animal feeding (Kerem and Hadar 1998). Additionally, this edible mushroom has served as a source for obtaining enzymes used in decoloring molasses and removal of phenols.

The use of wastewaters for submerged fermentation in obtaining primary inoculum, permits the utilization of the residue and the reduction of organic load. Also, submerged cultures reduce some of the problems found in the traditional method of cultivation (e.g. relatively long time in the production of secondary inoculum (32-34 days), frequent contamination during substrate manipulation, and difficulties in the control of the process) (Nieto and Sánchez 1997).

Determining optimal conditions for growth of *P. ostreatus* on wastewater from mushroom farms for pollution reduction was the objective of the present study.

MATERIALS AND METHODS

Effluent

CPW were collected from the CEBI mushroom farm, filtered through gauze to eliminate solid materials, centrifuged at 3000 rpm for 5 min, and stored in plastic flask at 4 °C.

Microorganism and culture conditions

Stock cultures of *P. ostreatus* (Var. florida cfr. CCEBI 3024) were maintained at 4 °C on potato-dextrose agar (Difco Laboratories). *P. ostreatus* mycelia were adapted to grow on CPW on agar plates containing potato dextrose broth and concentrations of CPW (20 %). Cell extracts were prepared from *P. ostreatus* cultures grown for 7 days on plates. Mycelium from each plate was harvested and added to an Erlenmeyer flask containing 50 ml of NaCl (1%) and then homogenized. Aliquots (5 ml) of the homogenate were used to inoculate 50 ml of liquid media.

The basal synthetic medium (BSM) used was a modification of basidiomycetes rich medium (BRM) reported by Schützendübel *et al.* (1999), which contained the following components (g/l): yeast extract 0.5, L - asparagina 0.65, KH₂PO₄ 1.0, KCl 0.5, MgSO₄ x 7H₂O 0.5. One milliliter of micro-elements solution (1000x) was added. These solutions contained in (mg/l): boric acid 500, CuSO₄ x 5H₂O 40, KI 100, FeCl₂ x 6 H₂O 200, MnSO₄ x 4H₂O 400, ZnSO₄x7H₂O 400, NaMBO₄ x 2H₂O 200. The pH of BSM was adjusted to pH 6. CPW was added to BSM at a concentration of 50 % in all experiments.

All of the Erlenmeyer flasks were placed on a rotary shaker at 120 rpm , and then incubated at 28 °C in the dark. Five replicates for each set of conditions and times were performed.

Growth conditions on CPW.

Three sources of carbon were used: glucose, sucrose and glycerol. Each one was added at a rate of 20 g/l.

The best carbon source was selected by evaluating biomass and CPW decolourisation levels. Different concentrations (5, 10, 20 g/l) were then assayed in order to select one to be used in the process. The selection of the concentration was based in the analysis of biomass and colour removal, along with the level of laccase enzyme activity.

The effects of the addition of certain ingredients to BSM for growth and colour removal with *P. ostreatus* were studied. Two experiments were performed in parallel: the first one employed the complete BSM, and the second one used a solution of microelements.

Decolorization test

Erlenmeyer flasks with 50 ml of CPW and BSM using glucose were inoculated. Some of the flasks were removed at ten days and the others were left for twenty days. The same parameters of previous experiments were observed including the chemical oxygen demand (COD).

Analytical methods

Dry weight for the evaluation of the biomass, luminance for colour measuring and COD were analyzed following standard methods proposed by the American Public Health Association (1989). Biomass yield was estimated after drying the mycelial samples at 105 °C for 24 h. The calculation of color removal was performed by means of the following equations:

$$R = (A_i - A_f) / A_i \quad (1)$$

$$A = 2 - \text{LOG } L \quad (2)$$

where:

L - luminance values

R - Color removal

A_i - Absorbance obtained from the value of initial luminance according to (2)

A_f - Absorbance obtained from the value of final luminance according to (2)

LOG - logarithm

Residual glucose in culture medium was determined according to Miller (1959) and carbohydrates following the technique reported by Dubois *et al.* (1956).

Enzymatic activity

Laccase activity was measured by the oxidation of guaiacol at 460 nm (Palmieri *et al.* 1997) in a reaction mixture (1 ml) at 30 °C that contained 10 mmol/l guaiacol and 50 mmol/l of phosphate buffer at pH 6. (Enzymatic activity is defined as the quantity of enzyme that produces an increment of absorbance of one unit/min.)

RESULTS

Selection of the carbon source and its concentration.

Guzmán in 1993 reported that *P. ostreatus* can use sucrose, manose, maltose, fructose, pectin, lignin, cellulose and starch as carbon sources for its mycelial growth. Glucose, sucrose and glycerol were selected for use during this experiment.

The highest values of biomass and conversion efficiency were obtained in the cases where glucose was used (Table 1). There was a reduction in growth with sucrose as substrate (lowest in the experiment - 6.16 g/l).

Table 1. Biomass, yield and color removal in coffee pulp wastewater by *Pleurotus ostreatus* using different carbon sources.

Treatment	Biomass (g/l)	Y x/s	% R (colour)
CPW + Glucose + BSM	13.27 ^a	0.66 ^a	66 ^a
CPW + Sucrose + BSM	6.16 ^b	0.31 ^b	50 ^a
CPW + Glycerol + BSM	10.77 ^{ab}	0.54 ^{ab}	66 ^a

The biomass was obtained as dry weight.

Values in the same column with the some letters are not statistically different at the 1% level.

Table 2. Influence of different glucose concentrations on biomass, yield and color removal in coffee pulp waste water by *Pleurotus ostreatus* CCEBI 3024.

Treatment	Biomass as dry weight (g/l)	Enzymatic activity (U/ml)	% R (colour)
CPW+5g/l	1.42 ^c	3.18 ^a	8.1 ^c
CPW+10g/l	4.37 ^b	2.19 ^a	23.3 ^b
CPW+20g/l	8.26 ^a	1.88 ^a	57.1 ^a

Dry weight increased with glucose concentration (Table 2) through values of 8.2 g/l at 20 g/l of glucose. These results are similar (although at ten days) to those obtained on synthetic medium (8.6 g/l) by Guillén *et al.* (1998) after 16 days of fermentation. The best yield biomass/substrate (0.8) was obtained at 10 g/l of glucose, compared with 0.3 and 0.4 obtained for 5 g/l and 20 g/l, respectively. Obtaining biomass this way can serve as an alternative to obtaining culture inoculum from solid media and permits adaptation for growth on coffee pulp residues.

There were no significant differences in color removal among the treatments because the carbon source used did not influence decolourization. Colour-removal exhibited the same results as did biomass, with the lowest value for concentrations 5 g/l. According to Pellinen *et al.* (1988), when the glucose is used as substrate for growth, a minimum concentration of 2 g/l is necessary for the mushroom to maintain its decolorisation ability. The highest removal levels were obtained at high concentrations of glucose (concentrations greater than 10 g/l).

Laccase activity has been observed in the decolourization of molasses and textile wastewaters (Knapp and Newby 1998). For these substrates the influence of carbon source concentration on the excretion of enzyme is known. In this study, no significant differences were found among the enzymatic activities at the various concentrations of the carbon source tested, although the enzyme activity was increased when the glucose concentration was lower (Table 2).

The effect of the addition of oligoelements of MSH on growth and decolourisation with *P. ostreatus*

Macro-elements of the BSM supplied CPW with the necessary quantities of nitrogen and salts for the growth of the mushrooms. No statistical differences were found in biomass, color removal or enzymatic activity between supplemented and un-supplemented CPW (Table 3).

Table 3. Effect of components on *Pleurotus ostreatus* culture.

Treatment	Biomass (g/l) as dry weight	% R (colour)	Enzymatic activity (U/ml)
CPW + BSM	13.27 ^a	66 ^a	2.19 ^a
CPW + microelements	11.80 ^a	60 ^a	2.30 ^a

Decolorisation assays

In the bibliographies consulted there are differences in enzymatic profiles and decolourising values, depending on the mushroom's age, the residue treated, and culture conditions. For this reason, an experiment on enzymatic activity, colour removal and the organic load (COD) with *P. ostreatus* was performed. Table 4 shows that all parameters have an increase at the end of 20 days of fermentation, except enzymatic activity. The reduction in the enzymatic activity could be attributed to the accumulation of mushroom byproducts.

Table 4. Effects of *Pleurotus ostreatus* culture on wastewater of coffee pulp.

Fermentation times	Enzymatic activity (U/ml)	Biomass (g/l) as dry weight	% R (COD)	% R (colour)
10 days	3.0	5.4	25	44
20 days	1.6	9.6	55	47

In the culture medium, mycelial growth occurs in pellet shapes, typical of the submerged culture with agitation, and absorption of colour was not observed on the mycelia.

The highest enzymatic activity was obtained at ten days of fermentation (3.0 U/ml), superior to the activity obtained on synthetic medium. Mushroom growth on BSM without CPW reached a maximum laccase enzymatic activity at 5 days (0.29 U/ml) and this value diminished through the tenth day of fermentation (0.14 U/ml).

As indicated in Table 4, the majority of the colour disappeared in the first ten days coinciding with the period of higher laccase activity. Thereafter, decolourising activity continues, but in a smaller proportion. Other studies have demonstrated certain correlations between the production of this enzyme by *Pleurotus* spp and industrial dye decolourisation (Rodríguez 1999). Shin *et al.* (1997) reported the existence of other white-rot mushroom enzymatic systems that contribute to the process of decolourisation, such as cytochrome P₄₅₀ or peroxidases.

In CPW most of the color disappeared during the first 10 days, but the colored compounds removed constitute less than half of the DQO that is removed during the whole treatment. It is possible to remove the organic load pollutant more than 50 % in 20 days of fermentation.

DISCUSSION

The CPW contains some antinutritional compounds, e.g. tannins, polyphenols and chlorogenic acid. However, during this experiment, a high biomass concentration was obtained using CPW (50 %). The best conversion efficiency values were obtained with glucose concentrations above 10 g/l. Glucose is, among the carbon sources tested, the most easily assimilated sugar. The addition of this sugar supplements the low levels of carbohydrates available for assimilation in CPW.

The basidiomycete culture medium used in this work (CPW+BSM) can be simplified by leaving out some ingredients of BSM, e.g. yeast extract, L-asparagine and some salts. CPW could support most requirements for *P. ostreatus* growth and for enzyme excretion. Besides, the media formed with CPW guarantees the nitrogen and sulphur conditions necessary to activate the ligninolytic system (Feijoo and Lema 1995).

Appropriate mushroom growth is necessary for color removal because this assures the synthesis and liberation of enzymes and mediators used in the decolorizing process. Among those enzymes, *P. ostreatus* produces laccase enzymes which have been demonstrated to be effective in degrading recalcitrant compounds, some of them associated with colour. These results also show that there are other enzymatic systems involved in the decolourising process in live cultures.

Some culture conditions, such as mycelium age and media composition, affect enzymatic activity levels. At the end of the fermentation process (20 days) laccase activity diminished. It was better in CPW than the media without wastewater. The profiles of enzymatic activity obtained in presence of CPW were similar to those of other studies of wastewater degradation with *P. ostreatus* (Kerem *et al.* 1992, Martirani *et al.* 1996). In these studies the laccase activity had an increment from the sixth day of fermentation and then fell to $\frac{1}{4}$ of the initial activity at 18 days. Maximum activity was obtained between 10-13 days of fermentation. With CPW, enzyme activity was achieved at 10 days compared with 5 days on synthetic medium. In this wastewater are phenols and other related compound such as caffeine (López *et al.* 1995) which could induce this enzymatic activity and maintain a larger fermentation period.

The cultivation of *P. ostreatus* reduces the pollution generated by coffee pulp wastewater, removing its organic load and colour. The COD removal values are similar to those reported by other authors (Vidal *et al.* 1996, Bello and Sánchez 1997). There is relatively little information about colour removal in wastewater from coffee pulp.

In conclusion, CPW can be employed to produce mycelia of *P. ostreatus* with good yields. Efficient removal values for colour and COD were obtained by cultivating this mushroom.

REFERENCES

- American Public Health Association. 1989. Standard methods for the examination of water and wastewater, 17th ed., Washington, USA.
- Bermúdez, R. C and J. L. Pérez. 1998. Biogás a partir de los efluentes de la pasteurización de la pulpa de café. In: *Proceed. CUBASOLAR 98*: 13-17.
- Bermúdez, R. C., N. García, P. Gross and M. Serrano. 2001. Cultivation of *Pleurotus* on agricultural substrates in Cuba. *Micología Aplicada Internacional* 13 (1): 25-29.
- Bello, R. and J. E. Sánchez. 1997. Anaerobic filter treatment of wastewater from mushroom cultivation on coffee pulp. *World Journal of Microbiol. & Biotechnol.* 13: 51-55.
- Dubois, M., K. Guilles, J. Hamilton, P. Roberts and F. Smith. 1956. Colourimetric determination of sugars and related substances. *Analyt. Chem.* 28: 350-356.
- Eggen, T. and A. Majcherczyk. 1998. Removal of polycyclic aromatic hydrocarbons (PAH) in contaminated soil by white rot fungus *Pleurotus ostreatus*. *Intern. Biodegrad.* 41: 111-117.
- Feijoo, G. and J.M. Lema. 1995. Tratamiento de efluentes de industrias de la madera con compuestos tóxicos y recalcitrantes mediante hongos. *Revista Afinidad LII*: 171-180.
- Guzmán, G. 1993. Studies of genus *Pleurotus* (Basidiomycotina) in Mexico and other Latin-American countries: Taxonomic confusion, distribution and semi-industrial culture. *Cryptogamic Botany*:220-321.
- Guillén, G., F. Márquez and J. Sánchez. 1998. Producción de biomasa y enzimas ligninolíticas por *Pleurotus ostreatus* en cultivo sumergido. *Rev. Iberoam. Micol.* 15: 302 – 306.

- Kerem, Z., D. Friesem and Y. Hadar. 1992. Lignocellulose degradation during solid-state fermentation: *Pleurotus ostreatus* versus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 58 (4): 1121-1127.
- Kerem, Z. and Y. Hadar. 1998. Lignin-Degrading Fungi: Mechanisms and utilization. *Agricultural Biotechnology* : 351-365.
- Knapp, J. S., P. S. Newby. 1999. The decolourisation of a chemical industry effluent by white-rot fungi. *Water Research* 33 (2): 575-577.
- López, Z., J. Sánchez and R. Bello. 1995. Coffee wastes: quality of effluent water from the pasteurizing of coffee pulp. In: *Proceedings of the 16th International Scientific Colloquium on Coffee*. ASIC. Kyoto: 440-446.
- Martirani, L., P. Giardina, L. Marzullo and G. Sannia. 1996. Reduction of phenol content and toxicity in olive oil mill wastewaters with the ligninolytic fungus *Pleurotus ostreatus*. *Water Research* 30 (8): 1914-1918.
- Miller, G. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal. Chem.* 31: 426-428.
- Nieto-López, C. and J. Sánchez-Vázquez. 1997. Mycelial growth of *Pleurotus* and *Auricularia* in agroindustrial effluents. *Micología Neotropical Aplicada* 10: 47-56.
- Palmieri, G, P. Giardina, C. Bianco, A. Scaloni, A. Capasso and G. Sannia. 1997. A novel white Laccase from *Pleurotus ostreatus*. *J. Biol. Chem.* 272 (50): 31301-30307.
- Rodríguez, E., M. Pickard and R. Vazquez. 1999. Industrial Dye Decolorization by Laccases from Ligninolytic Fungi. *Current Microbiology* 38: 27-31.
- Shin, K., I. Oh and C. Kim. 1997. Production and purification of Remazol Brilliant Blue R Decolorization peroxidase from the culture filtrate of *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* 63: 1744-1748.
- Shüttendüel, A. and A. Majanherczk. 1999. Degradation of fluorene, phenanthrene, fluoranthene, and pyrene lacks connection to the production of extracellular enzymes by *Pleurotus ostreatus* and *Bjerkandera adusta*. *International Biodeterioration* 43: 93-100.
- Vidal, G., R. Méndez and J. Lema. 1996. La industria de pasta celulósica y papel: Tratamiento externo de los efluentes residuales. *Rev. Ingeniería Química* 6: 139-146.