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Obtaining and characterizing *Pleurotus ostreatus* strains for commercial cultivation under warm environmental conditions

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ABSTRACT

Pleurotus ostreatus strains obtained by interbreeding were cultivated under warm conditions (27 °C), with the aim to identify germoplasm adequate to commercial production. Based on the radial growth rate of monokaryotic mycelia isolated from two *P. ostreatus* strains, a culture isolate was selected from each type of compatibility, and interbred to obtain 16 strains. Pasteurized barley straw was used as the substrate for mushroom production. The mean biological efficiency of the strains was 84.17% and the production rate was 1.76%. *In vitro* laccase activity was tested for both parental strains and the four most productive strains of progeny. The mean enzyme activity of the strains was 1.21 μg mol/disc/ABTS oxidized. These results show the high potential of these strains for their use in substrate delignification and the production of basidiocarps under natural environmental conditions of warm regions.
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1. Introduction

From 1995 to 2001 commercial mushroom production in Latin America is estimated to have increased 32%, from 49,975 t to 65,951 t per year. Production was led by Mexico (58.6%), Chile (17.6%) and Brazil (10.6%), for 86.8% of the total mushroom production. *Agaricus* and *Pleurotus*, the most commonly grown mushrooms, represent an estimated 95% and 5% of production, respectively (Martínez-Carrera, 2002). The production of *Pleurotus* in Mexico was 4380 t in 2002 (Lahman and Rinker, 2004), and this has increased by 5000 t since 2005 (Gaitán-Hernández et al., 2007).

Pleurotus species, commonly known as oyster mushrooms, are edible fungi that are cultivated worldwide. The genus is characterized by its high protein content and gourmet food quality, which surpass those of many other foods (Manzi et al., 1999). For the commercially cultivated species, the production system and environmental conditions such as temperature, humidity, light and ventilation are all factors that affect mushroom development and are variable. To date, *Pleurotus* species that can grow at high temperatures, such as *Pleurotus pulmonarius* (Fr.) Quél., *Pleurotus tuber-regium* (Fr) Sing. and *Pleurotus djamor* (Fr.) Boed., have received special attention by producers and are being promoted mainly for cultivation in the tropics (Isikhuemhen and Okhuoya, 1996; Gaitán-Hernández and Salmones, 1999; Salmones and Durán-Barradas, 2001). That said, *Pleurotus ostreatus* continues to be the species for which commercial demand is highest in these regions.

P. ostreatus is mainly cultivated on different types of cereal straw, and gives the best yield in temperate environmental conditions. This puts tropical countries at a disadvantage, since their high temperatures limit optimal production for most of the commercial strains that are available for cultivation under temperate conditions. *P. ostreatus* does not grow naturally in tropical regions and this limits the availability of genetic material (Guzmán, 2000). For these reasons, in this study *P. ostreatus* strains obtained by selective breeding were cultivated with the aim of identifying those with suitable commercial characteristics for culture in warm environments.

Another interesting feature of *P. ostreatus* is its laccase production. This oxidase is an important enzyme in the biodegradation of lignin and its derivatives, as well as in the detoxification of the xenobiotic compounds present in the majority of industrial residues. The variation in the production of this enzyme among the strains of this species has been widely documented (Medeiros et al., 1999; Arora and Gill, 2000; Hublik and Schinner, 2000). A direct relationship between the capacity for the secretion of this enzyme and the potential of colonization of the fungus on the substrate has yet to be established, but laccase does participate in the adaptation of the fungus to the substrate on which it grows. As such, we included the determination of the enzyme in culture media as an indicator of the degree of

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adaptation of the strains to the cultivation conditions being evaluated.

2. Materials and methods

2.1. Strains and growth conditions

P. ostreatus (Jacq.: Fr.) Kumm. strains used in the present study are deposited in the Fungus Strain Collection of the Institute of Ecology (Xalapa, Mexico). Strain IE-38 (=P1-27) was donated by Dr. S.T. Chang and strain IE-126 (=DR-93), from the southeastern USA, was provided by Dr. D.L. Ritcher of the Technological University of Tennessee, USA. Both strains were maintained on malt extract agar (MEA) (BIOXON, USA) at 27 °C.

2.2. Single sporing and crosses

Monokaryotic mycelia were isolated from basidiospore suspensions at a concentration of 1×10^6 spores per ml. The spore print was obtained from the basidiocarps of strains IE-38 and IE-126, previously developed on barley straw (*Hordeum* sp.). Aliquots (1 ml) of the basidiospore suspensions were spread on MEA in Petri dishes (90 mm Ø). After 3–4 days of incubation in darkness at 27 °C, the spores germinated and the monokaryotic mycelium was microscopically confirmed by the absence of mycelial clamp connections. Twenty monokaryotic mycelia were isolated for each strain.

Mating type was determined by randomly selecting 12 of the 20 original monokaryotic isolations. These 12 single spore cultures were paired in every possible combination, avoiding reciprocal crosses (Eger, 1978). Concurrently, mycelial growth on MEA was estimated for the 12 isolated single spores over 14 days of incubation (in darkness at 27 °C). Mycelial growth was estimated as radial growth rate (u_r, μm h^{-1}). Using these results, the fastest growing strains (u_r) were selected and with these, interstock crosses were carried out. Dikaryotization was confirmed on observing mycelial clamp connections in the area of contact between the monokaryotic mycelia. The u_r and primordia formation of the new dikaryons were registered. This was done by placing the crosses developed on MEA in Petri dishes, in a humid chamber to induce primordia formation. In addition, early fruiting was evaluated on small samples of pasteurized barley straw (500 g wet weight). These samples were inoculated (5%, w/w) and incubated in darkness at 27 °C until the primordia formed. Eight of the 16 strains obtained had the best radial growth rate and early fruiting, and were selected for fruiting body production.

2.3. Fruiting and cultivation methods

The spawn was prepared with sorghum seeds (*Sorghum bicolor* L. Moench), adjusted to *ca.* 55% moisture (*ca.* 400 g wet weight), placed in heat resistant polyethylene bags and sterilized at 121 °C for 1 h. After cooling, the seeds were inoculated with the mycelium from each strain of *P. ostreatus* (Gaitán-Hernández et al., 2004).

For the production of fruiting bodies, prior to use the barley straw was fragmented into small pieces (5–8 cm long) with an electric chopper. It was then pasteurized at 65 °C for 1 h. Sowing was carried out using a mixture of pasteurized straw (65% moisture) and spawn from each of the strains was placed in transparent polyethylene bags (40 cm × 60 cm). In each bag, the wet weight equivalent of 500 g dry weight of straw was inoculated to 5% (w/w). These were incubated in the dark at 27 °C until the primordia appeared (11–18 days), at which point the samples were placed in favorable conditions of natural light (12 h light/12 h darkness), with forced ventilation (equal to 12 changes/h) to keep CO_2 levels low (<1200 ppm), 85–90% relative humidity and a temperature of 27 °C. The mushrooms were harvested at the adult stage, when the pileus was completely extended. Productivity was expressed as biological efficiency (BE) (fresh weight of mushrooms harvested/dry weight of substrate used × 100) and production rate (PR) (% biological efficiency/days elapsed between inoculation and final harvest). The harvested mushrooms were also classified according to the size of the developed pileus: group 1 (G1) <5 cm, group 2 (G2) from 5 to 10 cm, and group 3 (G3) >10 cm.

2.4. In vitro laccase activity

Inocula of each strain were prepared on MEA. After 7 days of incubation, discs of mycelium (7 mm ∅) from the inocula were placed in Petri dishes on agar and incubated for 7 days in darkness at 27 °C.

Laccase activity (EC 1.10.3.2) was evaluated by applying the technique of Niku-Paavola et al. (1990), modified by Mata et al. (1997). This consists of taking a disc of mycelium (7 mm ∅) from the edge of the Petri dish for each *Pleurotus* strain. This is then placed in a tube with 1.5 mL of 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) at 2%, dissolved in sodium acetate buffer 0.1 M, pH 5. Samples were incubated for 30 min at 30 °C. The concentration of the recovered ABTS solution was read at 426 nm. One unit of laccase activity (U) was expressed as 1 μmol of ABTS oxidized per minute per mycelial disc, and was calculated using the molar absorbency value of E_{426} = 29,300 M^{-1} cm^{-1}.

2.5. Experimental design and statistical analysis

A completely randomized design with a factorial arrangement was applied to radial growth rate and to the enzyme activity values; five samples were evaluated per strain. A completely randomized design with a factorial arrangement was also applied to production values; 12 samples were evaluated per treatment. An analysis of variance was done for all values and Tukey's test was used for the comparison of means ($p < 0.05$) using the Statistica version 7.0 statistical package.

3. Results and discussion

3.1. Strains produced from interstock crosses of IE-38 & IE-126

As a result of interbreeding the 12 monokaryotic cultures of the parental strains, 1–5 monokaryons were grouped into four mating types classes (Table 1), since *P. ostreatus* has a tetrapolar heterothallic sexual pattern (Eugenio and Anderson, 1968). The mycelial radial growth rate of IE-38 monokaryons ranged from 271.87 to 468.75 μm h^{-1}, requiring 11 days to cover the surface of the medium, while the u_r of IE-126 monokaryons was 142.70–391.66 μm h^{-1}, and the mycelia covered the surface of the medium after 14 days of incubation (Table 1).

During dikaryon evaluation, the u_r of the crosses was 684.37–758.33 μm h^{-1}, and for the parental strains it was 717.7 (IE-126) and 759.37 μm h^{-1} (IE-38) (Table 2). These results allowed us to choose the crosses for fruiting body production.

A mean of 6 days was required to cover the surface of the medium for all strains. No significant differences ($p > 0.05$) were observed between the radial growth rate of 11 crosses and parental strain IE-38. Parental strain IE-126 was statistically similar ($p < 0.05$) for all strains (Table 2). All strains exhibited hyphal aggregation in the humid chamber after 8 days of incubation; in the small samples of substrate eight crosses (C1, C7, C10–C13, C15, C16) displayed pinhead formation before 15 days of incubation, and the other strains before 16–20 days of incubation. Early

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