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Production of *Agaricus bisporus* on substrates pre-colonized by *Scytalidium thermophilum* and supplemented at casing with protein-rich supplements

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ABSTRACT

The objective of this study was to evaluate performance of *Agaricus bisporus* (*Ab*) on substrates pre-colonized by *Scytalidium thermophilum* (*St*), a thermophilic fungus known to enhance yields of *Ab* and increase selectivity of the substrate. The radial extension rate (RER) of the mycelium of three strains of *St* and their influence on the growth of a brown strain of *Ab* were evaluated. We also determined the time required for colonization of pangola grass by *St* in a compost pile and the influence of three protein-rich supplements on yield of *Ab* on pangola grass (*Digitaria decumbens*) colonized by *St*. RER of *St* ranged from 10.1 mm/d on grass to 18.9 mm/d on potato dextrose yeast extract agar, with significant differences among substrates and among strains. *Ab* grew faster on substrate colonized for 1, 2, or 3 days by *St* (RER of 3.31, 3.29, 3.23 mm/d, respectively) compared to non-colonized substrate (1.85 mm/d). *Ab* was cultivated on substrate samples selected daily from the *St*-inoculated pile, with biological efficiencies (BE) ranging from 4% (day 0) to 73.9% (day 2). Protein-rich supplements (soybean, black beans and cowpeas) added at casing significantly stimulated mushroom yield on *St*-colonized substrate compared to the non-supplemented control. BE varied from 26.1% on substrate non-supplemented to 73.1% on compost supplemented with ground soybean. There were no significant differences in mushroom yield observed among supplements evaluated.

1. Introduction

Commercial production of *Agaricus bisporus* is dependent upon the use of a composted mixture of wheat straw, hay, cottonseed hulls, seed meals, horse and poultry manure, gypsum, or other raw materials. Composting is accomplished in a two-phase process. Phase I involves mixing and wetting the raw materials as they are formed into a pile. Aerobic fermentation begins as a result of the growth and reproduction of microorganisms that occur naturally in the bulk ingredients. Phase I composting may last from 6 to 14 days, depending on the nature of the raw materials and their characteristics at each turn (Royse and Beelman, 2007). Phase II composting is conducted indoors and involves pasteurization to eliminate unwanted pests and removal of ammonia formed during Phase I composting. The production of Phase II compost requires 6–14 days depending on the system used. Properly finished compost is highly selective for growth and development of mushrooms.

The preparation of compost requires substantial space, time, labor and investment in equipment. In addition, there is a loss of substrate raw materials during composting and there may be negative environmental impacts such as the generation of offensive odors and effluents produced during the composting process (Straatsma et al., 1994b).

Because of these limitations, several researchers have investigated alternative methods of mushroom production on non-composted substrates (Till, 1962; Sanchez and Royse, 2001; Sanchez et al., 2002; Garcia et al., 2005; Bechara et al., 2005, 2006). These studies demonstrated that it is possible to obtain biological efficiencies ranging from 90% to 200% in three breaks; however, the main disadvantage is the cost of raw materials such as grain and for energy required to pasteurize/sterilize the substrate. These methods currently have a higher economic threshold compared to the traditional method, limiting their commercial use.

Recently our laboratories have developed a simplified method to cultivate *A. bisporus* without Phase I outdoor composting and with lower energy input. Colonization of mechanically ground substrate by the thermophile *Scytalidium thermophilum* (*St*) before spawning the mushroom (Sanchez et al., 2008) improved yields as compared to substrates non-colonized by *St*. This method requires temperatures of only 60 °C for pasteurization of the raw materials before inoculation of *St*. However, yields have been rather low (48.1% BE) compared to traditional compost. Therefore, the objective of this research was to examine additional factors,

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such as time of composting and supplementation of substrate at casing that might increase biological efficiency.

2. Methods

2.1. Isolates and culture media

Isolates of *A. bisporus* (Brown ECS-0305) and *S. thermophilum* (ATCC 66938, ATCC 22081 and ATCC 22082) were used. The medium for maintenance of all fungi was potato dextrose agar + 0.3% yeast extract (Difco) (PDYA). A starch medium (SA) was prepared with yeast extract (0.3%), potato infusion (20% from 200 g potatoes boiled in 1 L water), starch (1.5%), $MgSO_4$ (0.05%), K_2HPO_4 (0.1%), agar (1.5%). The media were adjusted to pH 7.5 with 0.1 N KOH and sterilized at 121 °C for 20 min.

2.2. Inoculum preparation

Mushroom spawn was prepared in 500 ml flasks with sorghum grain moistened to 50%, mixed with 2% $CaCO_3$ and 1% $CaSO_4$ (Fermont, Productos Quimicos de Monterrey, S.A. de C.V.), and sterilized at 121 °C for 20 min. Incubation after inoculation was at 25 °C for 10 days. The *St* inoculum was prepared with rice grain (pre-cooked rice SOS, S.A. C.V. Veracruz, Mexico), mixed with 2% $CaCO_3$, moistened to 50% and heat-treated at 110 °C for 15 min. The inoculated grain was incubated at 45 °C for 4 days.

2.3. Substrate preparation

For mycelial growth of the three *St* strains, pangola grass was coarsely ground (2–2.5 cm) with a semi-industrial mill (Inisa model TH2500), mixed with 2% hydrated lime (82% $Ca(OH)_2$, Super Calhidra Cales y Morteros del Grijalva, Mexico) moistened with tap water (70%) and autoclaved at 121 °C for 30 min. Pangola grass, recently harvested, was purchased from the local market and stored until needed with a maximum storage time of <4 months. For production trials, the substrate was pasteurized using two different methods: (a) in 1 kg bags placed in an incubator (Lab-Line Instruments, Model 3525, Melrose Pk, IL) for 8 h at 60 °C; or (b) in batches of 50 kg (70% moisture) contained in a wooden box (Tepemixtle, *Ocotea* sp.) of 75 cm per side with 50 holes 0.65 cm diameter at the bottom and a wooden cover on the top (Hernandez et al., 2003). Steam was applied underneath the substrate with a steam generator (Webeco GmbH, Bad Schwartau, Germany) to maintain a pasteurization temperature of 90 °C for 1 h. The substrate (50 kg) was cooled to 45 °C and seeded with *St* inoculum (0.3% wet wt basis). Incubation for growth of *St* was carried out in a self-heated compost pile 40 cm deep, placed in the wooden box described above. The substrate was removed from the box and tumbled for 30 s every 2 days in a mixer (Mezcladora MX-250, Xalapa, Mexico) for 5 days.

2.4. Cultivation methods

After colonization by *St*, substrates (950 g) were mixed with 50 g *Ab* spawn and placed in 1 kg polyethylene bags (30 × 40 cm). To allow gas exchange, the upper end of the plastic bag was fitted into a plastic cylinder (4 cm diameter) and the hole covered with a clean white paper towel. Incubation lasted 3 weeks at 25 °C and after the substrate was colonized by the mushroom mycelium, the bag was opened and a casing (peat:lime:water 1:1:4; no thermal treatment) overlay (4 cm deep) containing casing inoculum (500 g/m^2) was applied. The substrate was further incubated at 16 °C and 90% relative humidity for 30 days. Irrigation was applied daily. Supplements, when used, were mixed into the substrate before application of casing. Mushrooms were harvested (three breaks) when the pileus was opened and the veil broken. No attempts were made to control weed molds and diseases.

2.5. Supplement preparation

Black beans, cowpeas, and soybean were purchased locally. They were ground (2 mm screen) and eight treatments were prepared with supplements singly or in mixtures. The supplements were autoclaved in bags (high density polyethylene bags, 30 × 40 cm) at 110 °C (pressure 70 kPa) for 15 min and mixed (6% dry wt) into the substrate prior to casing.

2.6. Treatments

For evaluation of the growth rate of *S. thermophilum*, three *St* strains were grown on three sterilized culture media (pangola grass + 2% hydrated lime [70% moisture], potato dextrose agar, and starch agar) contained in Petri dishes. To determine the influence of composting time on the growth rate and mushroom yield of *Ab*, one compost pile was prepared and samples (three replicates) collected daily for each treatment for days 0–5. For supplementation trials, substrate pasteurization was carried out for 8 h at 60 °C and *St* incubation performed in bags in an incubator at 45 °C (substrate non-composted). After spawn run of *Ab*, three supplements (ground soybean, cowpea, and/or black bean) were used alone or in mixtures applied at time of casing. The *Ab*-colonized substrate was fragmented and the ground supplements were through-mixed into the substrate at 6% of the dry wt.

2.7. Sampling

Each day during the composting process (days 0–5), two 950 g samples were collected from the center of the substrate (from each wooden box or replicate), allowed to cool to ambient temperature, and inoculated with 50 g *Ab* spawn. Methods for mushroom production on the substrate samples were as described above. An extra 250 g sample was collected to determine moisture, pH and *Ab* growth rate.

2.8. Radial extension rate (RER)

Autoclaved, sterile substrate (15 g) was placed aseptically in a Petri plate (90 × 18 mm) containing one grain of inoculum (12 replicates/treatment). Spawn grains were carefully selected for size uniformity. The substrate was incubated in the plates at 25 °C for 20 days (for *Ab*) and 45 °C for 4 days (for *St*). The growth of the colony was measured every 5 days for *Ab* and daily for *St*. The radial extension rate (RER) was estimated as follows: $RER = (X_2 - X_1/t_2 - t_1)$ where X is colony radius (mm) and t is time (days).

2.9. Biological efficiency (BE) and yield

BE was determined as the ratio of fresh mushrooms harvested (kg) per kg dry substrate and expressed as a percentage. Mushroom yield was expressed as kg/m^2.

2.10. Experimental design and statistical analysis

The comparisons of radial growth rate of *St* were made using a 3 (strains) × 3 (media) factorial design with 12 replicates. The composting experiment was replicated three times and the production trials had at least six replicates. For the production assays during composting and supplementation, a completely randomized design was used. Analysis of variance and mean separation were