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Working Title of Contribution: "Cultivation of Culinary–Medicinal Lion's Mane Mushroom ***Hericium erinaceus*** (Bull.:Fr.) Pers. (Aphyllophoromycetideae) on Substrate Containing Sunflower Seed Hulls"

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Cultivation of Culinary–Medicinal Lion's Mane Mushroom *Hericium erinaceus* (Bull.:Fr.) Pers. (Aphyllophoromycetideae) on Substrate Containing Sunflower Seed Hulls

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ABSTRACT: The mycelial growth rates in linear growth assays, yield, and mushroom productivity of *Hericium erinaceus* were evaluated in a substrate containing sunflower seed hulls as the main energy and nutritional component, with the addition of different levels of Mn(II) and/or NH_4^+. The mycelial growth rate in substrates possessing different sunflower seed hull sizes with or without the addition of wheat bran showed that, irrespective of the presence of wheat bran, higher mycelial growth rate was observed with the larger sunflower seed hull size (as disposed of by the regional oil-seed factory without additional process). Adding growth-limiting mineral nutrients such as Mn(II) (20 or 100 ppm) and/or NH_4^+ (200 or 500 ppm) increased the mycelial growth rate by 8%–16%. The first flush occurred at day 10 and the second at day 30, with a production cycle duration of 55 days starting from inoculation. No statistical differences were detected between accumulated biological efficiencies coming from different substrate formulations with the addition of wheat bran, barley straw, or poplar sawdust compared to the sunflower seed hull control, but a tendency for higher yield was observed for the substrate supplemented with 20 ppm Mn and 200 ppm NH_4^+. Sunflower seed hulls without supplementation constitute a very good basal substrate, so this substrate by itself constitutes a very good source of energy and nutrition for *H. erinaceus* growth and development.

KEY WORDS: *Hericium erinaceus,* mushroom cultivation, mushroom productivity, sunflower seed hulls, culinary–medicinal mushroom

INTRODUCTION

Hericium erinaceus (Bull.:Fr.) Pers. is an edible and medicinal basidiomycete that belongs to the Hericiaceae family (Hericiales). This mushroom is very popular in traditional Eastern medicine and has been used to treat different human illnesses. For this reason it has received considerable attention, and some of its bioactive substances and properties have already been studied (Mizuno, 1995, 1999; Park et al., 2002; Wasser, 2002; Wang et al., 2005). Different isolated compounds from the fruit body possess

ABBREVIATIONS

BE: biological efficiency; **LGT:** linear growth test; **MP:** mushroom productivity; **RH**: relative humidity; **SSH:** sunflower seed hulls.

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cytotoxic effects or activities on cancer cells (HeLa cells), stimulate activity of nerve growth factor synthesis, and have nematicidal and antimicrobial activities (Mizuno et al., 1992; Kawagishi et al., 1994, 1996; Stadler et al., 1994). It was reported that preparations from its fruit bodies and mycelia are useful in treating gastric ulcers (Yang, 1986). Moreover, the fruit of *H. erinaceus* or its broth from liquid cultivation exhibit antitumor activity (Kim et al., 2000; Park et al., 2002).

The therapeutic possibilities of this specialty mushroom focused our attention on the need for its cultivation at low cost on locally abundant lignocellulosic material such as sunflower seed hulls. *H. erinaceus*, like other basidiomycetes, can degrade the major components of wood—that is, lignin, cellulose, and hemicellulose—by producing enzymes that oxidize lignin as well as other enzymes that hydrolyze cellulose and hemicellulose (Zadrazil, 1985; Buswell and Oider, 1987).

A great variety of substrates are appropriate for *H. erinaceus* cultivation, such as sawdust, sugarcane bagasse, corncobs, and cottonseed hulls supplemented with rice or wheat bran (Oei, 1996). Cotton seed hulls are considered by some authors to be the best material for cultivating *Hericium* spp. because of its low cost, easy preparation, and high yield (Liu, 1981); it has been recommended for cultivating *Hericium* spp. in any cotton-growing region. Sunflower seed hulls are an abundant byproduct of edible oil production in Argentina. However, sunflower seed hulls have not been studied for cultivation of *Hericium* species.

Because substrate formulations containing sunflower seed hulls as the main lignocellulosic ingredient have been developed previously for *Lentinus edodes* (shiitake), *Ganoderma* spp., and *Pleurotus ostreatus* (oyster mushroom) production (Darjania et al., 1997; Curvetto et al., 1998, 2002a,b; González Matute et al., 2002), we can analyze the possible use of this material for commercial *H. erinaceus* cultivation.

Blanchette (1984) suggested that Mn(II) is an important component in the selective delignification of wood by white-rot basidiomycete fungi. An increase in the Mn(II) content of substrates increased the level of ligninolytic enzymes and lignin degradation by *P. ostreatus* (Kerem and Hadar, 1995). Kaal et al. (1995), in their study related to the activity of ligninolytic enzymes with several basidiomycetes, showed that mycelial growth in a medium with a high content of NH_4^+-N (approximately 800 ppm) was improved compared to a substrate with a low content (approximately 30 ppm); all the assayed basidiomycetes had manganese–peroxidase activity, which consistently increased with higher levels (56 mM) of peptone-N and NH_4^+-N. However, caution should be exercised with N substrate supplementation. It has been reported that N supplementation of decontaminated substrate that contained a high quantity of carbohydrates can stimulate dramatic growth of molds and competitive bacteria, producing a temperature increase (thermogenesis) sufficient to kill the mycelia of the oyster mushroom in less than 1 day (Lelley and Janßen, 1993).

The purpose of the present study was to evaluate the feasibility of using sunflower seed hulls as the main substrate for the production of *H. erinaceus* fruit bodies and to determine the value of nitrogen and/or manganese supplementation to improve its production.

MATERIALS AND METHODS

Microorganism and Cultivation Media

The *H. erinaceus* strain used was graciously provided by D. Rinker, University of Guelph (Ontario, Canada), and was maintained on sterilized MYPA medium (20 g malt extract, 2 g yeast extract, 1 g peptone, 20 g agar, and 10 g glucose per liter, pH 6) in tubes at 4°C until use. The mycelium was cultivated in the same medium in Petri dishes. Cultures were incubated in darkness at 25ºC for 14 days.

Substrate Formula Evaluation Using a Linear Growth Test

The linear growth test (LGT) described by Duncan (1997) was used to evaluate the mycelial growth rate of this mushroom on different substrates at the same density, as follows: (1) Sunflower seed hulls (SSH) of three average sizes (12×4 mm, 9×2 mm, and powder). The largest is the average size of seed hulls

obtained from oil-seed factories; 9 mm seed hulls were obtained by separation of ground hull mixture through a metal sieve with 9 mm mesh; and the powder was obtained using a Wiley mill sieve # 40 mesh. (2) SSH in those same sizes plus commercial wheat bran powder.

The basal formula contained (w/w) 37.5% SSH, 0.5% $CaCO_3$, 2% $CaSO_4$, and 60% tap water. In the formula including wheat bran, 2 parts (w) of SSH were replaced by 2 parts (w) of wheat bran, resulting a 8:2 SSH:wheat bran ratio.

Glass tubes 200 mm long and 16 mm in diameter were filled with 10 g of the substrates to a density of 0.5 g cm^{-3} and autoclaved for 120 minutes at 120ºC. A mycelium disk cut from a culture in the Petri dish was aseptically inoculated on one substrate end of each tube. Incubation proceeded at 25ºC in darkness. The linear mycelium growth rate was determined at day 12 (n = 10).

LGT for SSH Substrates Containing Different Content Ratios of $NH4^+$ and/or Mn(II)

This test was used to evaluate the rate of mycelial growth in a substrate containing SSH, manganese, and/or nitrogen in the form of NH_4^+. A factorial design was used with the basal substrate (SSH, 37.5%; tap water, 60%; $CaCO_3$, 0.5%; $CaSO_4$, 2%) in the absence or presence of Mn(II) or NH_4^+ or their combination. The $MnSO_4$ or $(NH_4)_2SO_4$ salts were dissolved in water and were added to the basal substrate to obtain the following treatment concentrations: 0, 20, 100, and 200 ppm of Mn(II); 0, 200, 500, and 750 ppm of NH_4^+; and all possible combinations of both salts. The linear mycelium growth rate was determined at day 7 (n = 10).

Spawn Production, Substrate Preparation, and Spawn Running

Spawn was essentially prepared as described elsewhere (Curvetto et al., 2004). Briefly, 1 L bottles were filled with 59.1% wheat grain, 40% water, 0.1% $CaCO_3$, and 0.8% $CaSO_4$ by weight. They were sterilized at 121ºC for 90 minutes and inoculated with *H. erinaceus* mycelium (two mycelium wedges per bottle). Inoculated grains were then incubated at 25ºC in darkness for 18 days with periodic inspection and shaking.

The following substrate formulations were assayed: SSH:wheat bran (8:2), SSH:powder malt extract (8:2), SSH:poplar sawdust (2 cm length) (8:2), SSH with addition of 200 ppm Mn(II) and 500 ppm NH_4^+, and SSH with addition of 20 ppm Mn(II) and 200 ppm NH_4^+. All formulations contained 60% water, 0.5% $CaCO_3$, and 2% $CaSO_4$ by weight.

The substrate formulations were aseptically inoculated with *H. erinaceus* spawn (8% by fresh weight), and 0.5 kg substrate was packed to an approximate 0.5 g cm^{-3} density into aseptically[[AU: "TREATED"?]] polypropylene bags (n = 10). Because a high CO_2 concentration—between 5000 and 40,000 ppm, according to Stamets (1993)—has to be developed to allow optimum mycelium growth, a small collar of approximately 13 mm diameter was inserted at the end of each bag and closed with cotton plugs.

To allow for complete substrate colonization, the bags were placed into a growth chamber at 24 ± 1°C, 95% relative humidity (RH), and darkness.

Fruiting and Crops

For a cycle production of *H. erinaceus*, bags with 500 g substrate were employed to study different formulas using SSH, in the absence or presence of 20% wheat bran, poplar sawdust, or malt or just in combination with Mn (II) and NH_4^+ in proportions that previously presented good performance in the LGT—that is, with the highest rates of mycelial growth. Substrate pH was 5.8 for the basal formula (SSH alone), and 5.5, 5.3, and 5.1 in the presence of poplar sawdust, wheat bran, and malt, respectively. When the mycelium of *H. erinaceus* completely colonized the substrate, the bags were removed from the growth chamber and punctured near the base, 2 holes (ca. 2 cm diameter) per bag, and placed in a controlled environment at 18 ± 1°C, 95% RH, and fluorescent white light (500–1000 lux, 12-hour cycle), with adequate ventilation to allow primordial induction. For subsequent fruit body growth, the room temperature was increased to

24°C. The biological efficiency (%BE = kg fresh mushrooms / kg dry substrate × 100) and mushroom productivity (MP = %BE / time of production cycle in days = kg fresh mushroom / kg dry substrate × 100 per day) were determined.

Experiment Design and Statistical Analysis

The LGT with different SSH sizes was done using 10 replications per treatment, and data were evaluated by ANOVA. Separation of mycelial growth rate mean values was performed by the Tukey test, $p < 0.05$. For LGT using substrates in the absence or presence of different combinations of Mn(II) and/or NH_4^+, 10 replications per treatment were done. Data were evaluated by the two-way ANOVA test, and the Dunnet test was used to separate control and treatment data ($p < 0.05$).

For fruiting evaluation, a completely randomized design with 10 replications per treatment was used, and results were analyzed by the ANOVA and Tukey tests, $p < 0.05$ for media separation.

RESULTS AND DISCUSSION

Substrate Formula Evaluation Using a Linear Growth Test

Table 1 shows the mycelial growth rate in substrates with different sizes of SSH with or without the addition of wheat bran. Irrespective of the presence of wheat bran, higher mycelial growth rates were observed with the largest SSH size, which were significant when compared with the powder form. This observation represents a clear advantage because no additional process is needed to use SSH to grow this mushroom.

LGT with SSH Substrates Containing Different Concentrations of $NH4^+$ and/or Mn(II)

Adding 20 or 100 ppm Mn(II) produced an increase of ca. 10% in the mycelial growth rate in the absence of NH_4^+; the same was true with either 200 or 500 ppm NH_4^+ or in other combinations of both salts—for example, 20 ppm Mn plus 200 ppm NH_4^+ and 200 ppm Mn plus 500 ppm NH_4^+ (Table 2). No effect on mycelial growth was observed in the case of 750 ppm NH_4^+. Unexpectedly, results with either 200/100 or 500/20 of NH_4^+/Mn showed no effect either.

Table 1: Mycelial Growth Rate (mm day^{-1}) of *Hericium erinaceus* Cultured in a Substrate Containing Sunflower Seed Hulls (SSH)*

Substrate	Mycelial growth rate mm day^{-1}**
(1)	3.3 **a** (0.32)
(2)	3.1 **ab** (0.10)
(3)	2.7 **c** (0.21)
(1) + WB	3.3 **a** (0.18)
(2) + WB	3.1 **ab** (0.11)
(3) + WB	2.8 **bc** (0.11)

* Three sizes were measured: (1) 12 × 4 mm, (2) 9 × 2 mm, and (3) powder, in the absence or presence of wheat bran (WB) (8 SSH : 2 WB, on dry weight basis). Values were measured at day 12.

** Different letters represent significant differences ($p < 0.05$) by the Tukey test. Standard error is given in parentheses.

Fruiting and Crops

The mycelium of *H. erinaceus* completely colonized the substrate in 25 days; at this time environmental parameters were modified for fruiting induction. Then 2 crops followed, with the first flush ending by day 10 and the second by day 30, with a whole production cycle duration of 55 days, starting from inoculation.

From the accumulated BE of different substrate formulations, it is possible to appreciate that the highest value was obtained from SSH supplemented with 20 ppm Mn and 200 ppm NH_4^+ treatment (43.1%), which was significantly different than 80 SSH + 20 poplar treatment; however, no statistical differences were detected with the remaining treatments (Table 3). The value of nitrogen and/or manganese supplementation to the SSH to improve the production of different *P. ostreatus* strains was studied by Curvetto et al. (2002a). In that case, a faster mycelial growth rate in LGT did not always lead to

Table 2: Mycelial Growth Rate of *Hericium erinaceus* (mm day^{-1}) Grown in a Substrate Containing Sunflower Seed Hulls (SSH) with Different Levels of Mn(II) and/or NH_4^+*

NH_4^+ (ppm) \ Mn(II) (ppm)	0**	20	100	200
0	2.4 **b** (0.16)	2.8 **a** (0.14)	2.8 **a** (0.08)	2.5 **b** (0.12)
200	2.7 **a** (0.09)	2.7 **a** (0.13)	2.4 **b** (0.26)	2.7 **a** (0.08)
500	2.7 **a** (0.16)	2.4 **b** (0.11)	2.6 **a** (0.12)	2.8 **a** (0.10)
750	2.4 **b** (0.11)	2.5 **b** (0.12)	2.4 **b** (0.17)	2.5 **b** (0.15)

* Mean values separated from control by Dunnet test ($p < 0.05$). Values were measured at day 7.

** Different letters represent significant differences ($p < 0.05$) by the Tukey test. Standard error is given in parentheses.

a higher yield in terms of biological efficiency, but it did when considering productivity.

Ko et al. (2005), in a comparative study of the production of different species of *Hericium* grown on oak sawdust substrate supplemented with rice bran in a ratio of 4:1 (w/w) in 1.1 L polypropyrene bottles, included just 1 strain of *H. erinaceus,* for which they found a biological efficiency of 31%, in a production cycle of 2 flushes (a third one could not be obtained), with a total duration from substrate inoculation until the last flush of 68 days. Because of the scarce information regarding *H. erinaceus* yield and considering that substrate weight described in Ko et al. (2005) was only twice that in our experimental design, we compare their results with our data. Ko et al. (2005) mushroom productivity resulted in ca. 0.5% per day. Considering the higher productivity value of ca. 0.7% per day obtained with *H. erinaceus* on the majority of the substrate formulas and overall cultural conditions assayed in the present work (Table 3), it is possible to conclude that SSH constitutes a very good basal substrate. It should be emphasized that SSH substrate containing no supplementation reached the same productivity as those obtained with N and Mn supplementation. It is concluded that these elements did not strongly limit *H. erinaceus* production under the above-mentioned conditions using SSH as the main lignocellulosic substrate.

Table 3: Accumulated Biological Efficiency (BE) and Mushroom Productivity (MP) of 2 Crops of *Hericium erinaceus* Grown on Various Sunflower Seed Hull (SSH) Substrate Formulations*

SSH (%)	Wheat bran (%)	Barley straw (%)	Poplar sawdust (%)	Manganese ppm	Ammonia ppm	Accumulated BE (%)	MP (%)/day
100	—	—	—	—	—	37.4 **ab****	0.7
80	20	—	—	—	—	37.2 **ab**	0.7
80	—	20	—	—	—	36.0 **ab**	0.7
80	—	—	20	—	—	33.0 **b**	0.6
100	—	—	—	200	500	38.0 **ab**	0.7
100	—	—	—	20	200	43.1 **a**	0.8

* Mushrooms were grown in 500 g polypropylene bags with SSH as the main substrate component, in the presence of some combinations of Mn (II) and NH_4^+ or in combination with one of the following additives: wheat bran, barley straw, or poplar sawdust. Total duration of the production cycle, 55 days.

** Different letters represent significant differences by the Tukey test ($p < 0.05$).

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