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Effect of edible oils and Cu (II) on the biodegradation of rice by-products by *Ganoderma lucidum* mushroom

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ABSTRACT

The biodegradation of rice agro-industrial residues (straw and husks) to obtain the medicinal white-rot fungi *Ganoderma lucidum* and a crude extract of laccases as by- product was studied. Mycelium growth analysis on substrate formulations in a solid state fermentation system showed a good growth performance on substrates containing 57–69% straw, 25–30% husk, 5–10% rice bran, and 0–1% olive oil. Total mushroom production of dried mushrooms increased from 2.9% to 3.7% when using 5% *vs.* 8% mycelium inoculation rates, and from 3.6% to 4.1% when 1% olive oil was added to the substrate formulation. Addition of 100 ppm Cu (II) as a substrate supplement increased laccase activity in mushroom residual substrate from 150 to 267 U/Kg substrate and produced a three-fold increase in mushroom copper content. Extracted laccase activity was stable after four months in freezer storage (−18 °C) and after 16 freezing/thawing cycles.

1. Introduction

Biodegradation of lignocelullosic wastes by white rot fungi is an energy-efficient process to produce edible and medicinal mushrooms while reducing the environmental impact which would otherwise be originated by an inadequate disposal. *Ganoderma lucidum* (Curtis: Fr.) P. Karst. (Reishi), a Basidiomycete of the family Ganodermataceae, is a medicinal mushroom with a broad spectrum of health benefits. The bioactive components found in this mushroom have numerous health properties to treat diseased conditions such as hepatopathy, nephritis, hypertension, hyperlipidemia, arthritis, neurasthenia, insomnia, bronchitis, asthma, gastric ulcers, atherosclerosis, leukopenia, diabetes, anorexia, and cancer (Batra et al., 2013).

Ganoderma lucidum is a white rot fungus usually grown on substrates made of a variety of crop and agro-industrial residues (González Matute et al., 2002, 2011). It uses three important enzymes, namely, laccase, (EC 1.10.3.2), lignin peroxidase (EC 1.11.1.14) and manganese peroxidase (EC 1.11.1.13) (Manavalan et al., 2012) to degrade lignin.

Rice straw, husks and rice bran are some of the most abundant agro-industrial by-products in the world. Some uses of rice straw and husks include forage and thermal–energy production; however these residues are mostly disposed of by burning them in open fields in a clearly non environmentally friendly practice (Lim et al., 2012). Other bioprocesses to treat rice by-products, like biofuel production, are under development. The great challenge in this field is to overcome the silica and lignin barrier to gain access to the carbohydrates (Zhang et al., 2012). In this sense, fungi have a tolerance to silica and some of them have the enzyme system required to degrade the lignin barrier. Moreover, some specialty mushrooms have been studied with the aim of recycling them into food as is the case of *Volvariella volvacea* (Kumhomkul and Panichpat, 2013) and *Pleurotus* spp. (Iqbal et al., 2005; Frimpong-Manso et al., 2011). Degradation of these rice by-products by other economically important species like *Lentinus edodes* has not been studied extensively (Ashrafuzzaman et al., 2009), nor has it been evaluated using *G. lucidum*.

In nature, *G. lucidum* grows as a parasite or a saprotroph on a wide variety of trees. Thus, it is expected that its mycelium should be able to cause a satisfactory degradation of a rice-based substrate, and if so, the process could eventually be improved by the addition of supplements or additives to overcome possible nutrient deficiencies as it occurred in the case of the bioconversion of spent

Abbreviations: B/A, biomass per area; BE, biological efficiency; SSF, solid-state fermentation; TMP, total mushroom production.

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rice straw substrate by the mushroom *Pleurotus sajor-caju* (Shashirekha et al., 2005). Among the possible substrate supplements, plant oils are of relevance due to their fungal growth stimulation properties. Particularly, fatty acid fractions from plant oils proved to stimulate the mycelium growth of *Agaricus bisporus* (Wardle and Schisler, 1969); sunflower oil increased the membrane permeability to the absorption of glucose in *Cordyceps militaris* (Park et al., 2002) and fruiting yields in *Pleurotus sajor-caju* (Shashirekha et al., 2002). Also, olive oil increased *Tricholoma matsutake* biomass (Guerin-Laguette et al., 2003) and increased polysaccharides production in submerged culture of *Grifola frondosa* (Hsieh et al., 2006). So far, the use of edible oils as substrate supplements to improve substrate degradation has not been evaluated for the cultivation of *G. lucidum*.

Management of agro-industrial residues usually lacks viability because of the high cost/income relation. In this study, apart from the production of *G. lucidum* mushrooms, two other possible additional profits are evaluated: the collection of spores released during maturation of fruiting bodies and the enzyme (laccase) extraction from the degraded substrate. In an indoor environment, spores of *G. lucidum* are deposited on surfaces of the environment where mushrooms grow. These spores possess highly bioactive metabolites, even higher than those contained in fruiting bodies, specifically certain fatty acids, triterpenes, and polysaccharides (Jin et al., 2013).

White rot fungi were used as biological agents in solid state fermentation processes to pre-treat rice by-products, not only aimed at the production of mushrooms but also at other possible applications like removal of lignin from the rice straw (Zhang et al., 2012) and enzyme production (Ruqayyah et al., 2013) among others. Hitherto, uses of residual substrate from *G. lucidum* cultivation have not been reported. Moreover those residues have not been used for enzyme extraction. Particularly, studies on laccase activity in mushroom residual substrates are scarce and limited to *Agaricus* spp. (González Matute et al., 2013) and *Pleurotus* spp. (Rinker, 2002).

This work deals with the study of a SSF process aimed at i) the cultivation of *G. lucidum* mushrooms using rice straw, husk and bran as main components to substrates and ii) the implementation of a low cost method for laccase recovery from the residual substrate. The stimulating effect of plant oils on mycelium growth and the effect of substrate supplementation with Cu (II) salts on mushroom yield and on laccase activity from residual mushroom substrate were also studied. Laccase extraction and freezing storage persistence of enzyme crude extracts from mushroom remaining substrates were also considered, since the recovery of this enzyme may lead to a more profitable mushroom production cycle. Because *G. lucidum* spores are considered to have medicinal value, a handling strategy for spore collection during the fruiting stage was also evaluated.

2. Materials and methods

2.1. Mushroom strain

Ganoderma lucidum E47 strain (University of Guelph, Canada) was cultivated in MYSA medium (20 g malt extract, 2 g yeast extract, 10 g sucrose, and 20 g agar per liter, pH 6) at 25 °C, in darkness.

2.2. Optimization of mycelial growth in agar nutrient media

Young mycelium of *G. lucidum* was inoculated into Petri dishes ($n = 7$) containing basal medium (MYSA, pH 6) with the addition of 0%, 1% or 5% (w/v) milled rice straw in combination with either sunflower or olive oil at concentrations of 0%, 1%, and 2% (w/w). Incubation was performed at 25 °C in darkness during 5 days. In addition, in order to evaluate a cheap and simple substrate formulation to be used by producers willing to prepare their own inoculum (spawn), the malt and yeast in MYSA medium were replaced by 40 g/L of commercial beer wort (*Dark Ale* variety from Coopers Brewery®). Hence, a second analysis of *G. lucidum* mycelium growth was made using beer wort -sucrose-agar medium (BwSA) under the same incubation conditions as the first one. Mycelium growth analysis was done as previously described (Postemsky et al., 2006). The mycelial growth rate was determined by measuring the diameter of colonization of the nutrient medium (as the mean of a pair of length diameters A and B, orthogonally measured and chosen at random) and by obtaining the biomass of dry mycelium after 5 days. Results are given as the dry mycelial biomass per unit area: $B/A\ (mg/cm^2) = dry\ biomass/[\pi \times (diameter\ A + diameter\ B)/4]^2$.

2.3. Lineal growth test

Mycelial growth on 24 different substrate formulations (Table 1) was evaluated using the lineal growth test of Duncan (1997). Each substrate (10 g) was packed to an approximate density of 0.5 g/mL in glass tubes (20 cm long and 16 mm width), 10 per treatment. Tubes were closed at both ends with cotton plugs, and sterilized in autoclave during 60 min at 121 °C. Substrate pH was determined before and after autoclaving. A 16 mm agar disk carrying actively growing mycelium of *G. lucidum* was placed at one end of each tube under aseptic conditions. Tubes were incubated at 25 °C and 80% RH in darkness for 8 days. Mycelium lineal growth was measured and the apparent mycelium density was scored as excellent (+++), good (++) or poor (+).

Further substrate selection for mycelium growth best performance was done by analyzing the increase in the soluble protein content in colonized substrates. Protein content was determined by using the dye-binding method of Bradford with bovine serum albumin as standard (Bradford, 1976). Briefly, sample aliquots (3.0 g fresh weight) were dispersed in 15 mL of 0.05 M potassium phosphate buffer (pH 7) and homogenized with pestle and mortar during 2 min. After centrifugation (10 min, 1,400*g*), 0.3 mL extracts were mixed with 2 mL of Bradford reagent, the reaction was allowed to proceed during 5 min and the absorbance at 595 nm was measured. Results were expressed as protein content per gram of colonized dry substrate (μg/g).

2.4. Inoculum production

Inoculum (spawn) was prepared according to Curvetto et al. (2004) using *Oryza sativa* grain (Double Caroline type). Incubation was performed at 25 °C in darkness for 10–15 days; bags were periodically shaken to minimize grain clumping and to allow for a better colonization.

2.5. Ganoderma lucidum production yield. Effect of olive oil and inoculum rate

The effect of olive oil on the production yield of *G. lucidum* at two inoculum rates was evaluated. Substrates containing 1% olive oil and control (treatments $L4_1$ and L4, respectively) were chosen after running the lineal growth test (2.4).

Substrate (20 kg) pasteurization was performed using a concrete mixer machine coupled with an oven gas burner (Curvetto et al., 2004). Once the threshold pasteurization temperature of 80 °C was reached in the substrate mass, a temperature between 80 °C and 90 °C was kept for an additional period of 60 min.

After cooling the substrate to 35 °C, the inoculum was added to, and mixed with, the substrate by drum rotation during 5–10 min.