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Biotechnology for *in vitro* growing of edible and medicinal mushrooms on wood wastes

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Abstract. The aim of this work was focused on finding out the best way to convert the wood wastes into useful food supplements, such as mushroom fruit bodies, by using them as growing sources for the edible and medicinal mushrooms. According to this purpose, three fungal species from Basidiomycetes, namely *Ganoderma lucidum* (Curt.:Fr.) P. Karst, *Lentinus edodes* (Berkeley) Pegler and *Pleurotus ostreatus* (Jacquin ex Fries) Kummer were tested to determine their biological potential to grow on substrates made of wood wastes (sawdusts as well as shavings) which could be used in this way as main ingredients for preparation of natural culture composts. The experiments were achieved by *in vitro* growing of all these fungal species in special rooms, where the main culture parameters were kept at optimal levels in order to get the highest production of mushroom fruit bodies. The effects of culture compost composition (carbon, nitrogen and mineral sources) as well as other physical and chemical factors (such as: temperature, inoculum amount, pH level and incubation time, etc.) on mycelial net formation and especially on fruit body induction, were investigated. From all these fungal species tested in our experiments, *P. ostreatus* was registered as the fastest mushroom culture, then *L. edodes* and finally, *G. lucidum* as the longest mushroom culture. During the experiments, different logs of the same species were used as control samples for each culture compost variants. Applying such biotechnology, the environmental problems generated by the plant wastes accumulation in wood industry could be solved only by using biological means for their valorising, simultaneously with food supplements producing having high nutritive values as well as healing effects by increasing the consumers` health.
Keywords: biotechnology, edible and medicinal mushrooms, wood wastes.

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Introduction

The most part of wastes produced all over the world arise from industrial, agricultural and domestic activities. These wastes represent the final stage of the technical and economical life of products (Verstraete & Top 1992). As a matter of fact, the forestry works, as well as the industrial activities related to forest management and wood processing have generally been matched by a huge formation of wide range of waste products (Beguin & Aubert 1994, Wainwright 1992). Many of these lignocellulosic wastes cause serious environmental pollution effects, if they are allowed to accumulate in the forests, or much worse to be

burned for uncontrolled domestic purposes. So far, the basis of most studies on lignocellulose-degrading fungi has been economic rather than ecological, with emphasize on the applied aspects of lignin and cellulose decomposition, including biodegradation and bioconversion (Carlile & Watkinson 1996). In this respect, the aim of this work was focused on finding out the best way to convert the wood wastes into useful food supplements, such as mushroom fruit bodies, by using them as growing sources for the edible and medicinal mushrooms (Smith 1998).

Materials and methods

Fungal species and culture media

According to the main purpose of this work, three fungal species from Basidiomycetes, namely *Ganoderma lucidum* (Curt.:Fr.) P. Karst, *Lentinus edodes* (Berkeley) Pegler and *Pleurotus ostreatus* (Jacquin ex Fries) Kummer were used as pure cultures in experiments. The stock cultures were maintained on malt-extract agar (MEA) slants. The slants were incubated at 25° C for 5-7 d and then stored at 4° C. The fungal cultures were grown in 250-ml flasks containing 100 ml of MEA medium (20% malt extract, 2% yeast extract, 20% agar-agar) at 23°C on rotary shaker incubators at 110 rev min^{-1} for 5-7 d.

Experimental methods

Preparation of liquid fungal inoculum

The fungal cultures for experiments were prepared by inoculating 100 ml of culture medium with 3-5% (v/v) of the seed culture and then cultivated at 23-25°C in rotary shake flasks of 250 ml. The experiments were conducted under the following conditions: temperature, 25°C, agitation speed, 90-120 rev min^{-1}; initial pH, 4.5-5.5. The seed culture was transferred to the fungal culture medium and cultivated for 7-12 d (Petre et al. 2005a, Glazebrook et al. 1992).

Incubation of mushroom cultures

The experiments were performed by growing all the previous mentioned fungal species in special culture rooms, where all the culture parameters were kept at optimal levels in order to get the highest production of fruit bodies. During the experiments, the effects of culture compost composition (carbon, nitrogen and mineral sources), as well as other physical and chemical factors (such as: temperature, inoculum size and volume and incubation time) on mycelial net formation and especially, on fruit body induction were investigated (Petre & Petre 2008).

All the culture composts for mushroom growing were inoculated using liquid inoculum with the age of 5-7 days and the volume size ranging between 3-7% (v/w). During the period of time of 18-20 after this inoculation, all the fungal cultures had developed a significant biomass on the culture substrata made of wood wastes, such as: white poplar and beech wood sawdusts. These wastes were used as main ingredients to prepare natural composts for mushroom growing. The optimal temperatures for incubation and mycelia growth were maintained between 23-25°C. The whole period of mushroom growing from the inoculation to the fruit body formation lasted between 30-60 days, depending on each fungal species used in experiments (Petre et al. 2007).

Preparation of mushroom culture composts

All these lignocellulosic materials were mechanical pre-treated to breakdown the lignin and cellulose structures in order to be more susceptible to the enzyme actions (Leahy & Colwell 1990, Petre et al. 2005b). All these pre-treated lignocellulosic wastes were disinfected by steam sterilization at 120^0 C for 60 min. The final composition of culture composts was improved by adding the following ingredients: 15-20% grain seeds (wheat, rye, rice) in the ratio 2:1:1, 0.7 - 0.9% $CaCO_3$, 0.3 -0.5% $NH_4H_2PO_4$, each kind of culture medium composition depending on the fungal species used to be grown. As control samples for each variant of culture composts used for the experimental growing of all these fungal

species were used wood logs of white poplar and beech that were kept in water three days before the experiments and after that they were steam sterilized to be disinfected.

Preparation of mushroom spawn

3000 g of white poplar sawdusts and 1500 g of beech sawdusts were mixed with cleaned and ground rye grain, 640 g of $CaCO_3$, 50 g of $NH_4H_2PO_4$ and 3550 ml of water, in order to obtain the growth substratum for mushroom spawn. The ingredients are mixed and sterilized at 121° C, for 20 minutes, and allowed to cool until the mixture is below 35° C. The spawn mixture is inoculated with 100-200 ml of liquid fungal inoculum. The materials are mixed for 10 min. to ensure complete homogeneity. The sterile polyethylene bags, containing a microporus filtration strip, are filled with the product and incubated at 25° C, until the spawn was fully colonized. At this point the spawn may be used to inoculate the mushroom growing substrate or alternatively it may be stored for up to 6 months at 4° C before use (Chahal & Hachey 1990). All the culture composts were inoculated using inoculum with the age of 5-7 days and the volume size ranging between 3-7% (v/w). The optimal temperatures for incubation and mycelia growth were maintained between 23-25°C. The whole period of mushroom growing from the inoculation to the fruit body formation lasted between 30-50 days, depending on each fungal species used in experiments.

Mushrooms cultivation

The experiments were carried out inside such *in vitro* growing rooms, where the main culture parameters (temperature, humidity, aeration) were kept at optimal levels to get the highest production of mushroom fruit bodies (Moser 1994). In order to find a suitable carbon source for the mycelia growth and, consequently, for fungal biomass synthesis, the pure cultures of *P. ostreatus* (Oyster Mushroom), as well as *L. edodes* (Shiitake) and *G. lucidum* (Reishi) were cultivated in different nutritive culture media containing various carbon sources, and each carbon source was added to the basal medium at a concentration level of 1.5% (w/v) for 7-12 d (Raaska 1990). To investigate the effect of nitrogen sources on mycelia growth and fungal biomass production, the pure cultures of these three fungal species were cultivated in media containing various nitrogen sources, where each nitrogen source was added to the basal medium at a concentration level of 10 g/l. In the same time, malt extract was one of the better nitrogen sources for a high mycelia growth. Peptone, tryptone and yeast extract are also known as efficient nitrogen sources for fungal biomass production by using the pure cultures of such fungal species (Chang & Hayes 1978). In comparison to organic nitrogen sources, inorganic nitrogen sources gave rise to relatively lower mycelia growth and fungal biomass production (Bae et al. 2000). The influence of various mineral sources on fungal biomass production was examined at a standard concentration level of 5 mg. In order to study the effects of initial pH correlated with the incubation temperature upon fruit body formation, *G. lucidum, P. ostreatus* and *L. edodes* were cultivated on substrates made of wood wastes of white poplar and beech at different initial pH values (4.5-6.0). The experiments were carried out for 6 days at 25°C with the initial pH 5.5. Similar observations were made by Stamets (1993), during the experiments. K_2HPO_4 could improve productivity through its buffering action, being favourable for mycelia growth. The experiments were carried out between 30-60 days at 25°C.

Results

The effects of carbon, nitrogen and mineral sources as well as other physical and chemical factors on mycelial net formation and especially, on fruit body induction were investigated by adding them to the main composts made of white poplar and beech sawdusts in the ratio 2:1. For the experimental growing of all these fungal species, white poplar and beech logs were used as control samples.

The effect of carbon sources upon the mycelia growth of pure mushroom cultures

When the cells were grown in the maltose

medium, the fungal biomass production was the highest among the tested variants (Table 1). Data presented in the following 6 tables are the means ± S.D. of triple determinations.

What it is very important to be noticed, it is that the maltose has a significant effect upon the increasing of mycelia growth and fungal biomass synthesis (Petre 2002). The experiments were carried out for 12 days at 25 °C with the initial pH 5.5.

The effect of nitrogen sources upon the mycelia growth of pure mushroom cultures

Among five nitrogen sources examined, rice bran was the most efficient for mycelia growth and fungal biomass production. The experiments were carried out for 12 days at 25 °C with the initial pH 5.5 (Table 2).

The effect of mineral sources upon mycelia growth of pure mushroom cultures

Among the various mineral sources examined, K_2HPO_4 yielded good mycelia growth as well as fungal biomass production and for this reason it was recognized as a favourable mineral source (Table 3).

Table 1 The effect of carbon sources upon the mycelia growth of pure mushroom cultures on white poplar and beech composts

Carbon source	Fresh fungal biomass weight (g/l)			Final pH		
	G. lucidum	*L. edodes*	*P. ostreatus*	*G. l*	*L. e*	*P. o*
Glucose	27 ± 0.10	41 ± 0.05	43 ± 0.03	5.5	5.3	5.1
Maltose	27 ± 0.14	45 ± 0.12	49 ± 0.05	5.8	5.4	5.3
Sucrose	25 ± 0.23	35 ± 0.03	37 ± 0.09	5.1	5.1	5.7
Xylose	26 ± 0.07	38 ± 0.07	35 ± 0.07	5.3	5.5	5.9

Table 2 The effect of nitrogen sources upon the mycelia growth of pure mushroom cultures on white poplar and beech composts

Nitrogen sources (1%, w/v)	Fresh fungal biomass weight (g/l)			Final pH		
	G. lucidum	*L. edodes*	*P. ostreatus*	*G. l.*	*L.e .*	*P. o*
Rice bran	37 ± 0.21	57 ± 0.05	73 ± 0.23	5.5	5.5	5.1
Malt extract	36 ± 0.12	55 ± 0.03	69 ± 0.20	5.3	5.2	5.7
Peptone	35 ± 0.03	41 ± 0.12	57 ± 0.15	4.6	4.9	5.3
Tryptone	36 ± 0.15	38 ± 0.07	55 ± 0.17	5.1	5.3	5.9
Yeast extract	37 ± 0.20	30 ± 0.01	61 ± 0.14	4.3	5.1	5.1

Table 3 The effect of mineral source upon mycelia growth of pure mushroom cultures on white poplar and beech composts

Mineral Sources (5 mg)	Fresh fungal biomass weight (g/l)			Final pH		
	G. lucidum	*L. edodes*	*P. ostreatus*	*G. l*	*L. e*	*P. o*
KH_2PO_4	37 ± 0.15	45 ± 0.07	53 ± 0.12	5.5	5.3	5.9
K_2HPO_4	45 ± 0.07	57 ± 0.05	59 ± 0.07	5.1	5.1	5.7
$MgSO_4 \cdot 5H_2O$	35 ± 0.25	55 ± 0.09	63 ± 0.28	5.6	5.4	6.1

The influence of initial pH and temperature upon mushroom fruit body formation

The optimal pH and temperature levels for fungal fruit body production were 5.0-5.5 and 21-23°C (Table 4).

To find the optimal incubation temperature for mycelia growth, these fungal species were cultivated at different temperatures ranging from 20-25°C, and, finally, the optimum of temperature was found at 23°C, being correlated with the appropriate pH level 5.5, at it is shown in Table 4.

The influence of inoculum age and inoculum volume upon mushroom fruit body formation

Amongst several fungal physiological properties, the age and volume of mycelia inoculum may play an important role in fungal hyphae development as well as in fruit body formation (Petre 2002).

To examine the effect of inoculum age and inoculum volume, mushroom species *G. lucidum, P. ostreatus* and *L. edodes* were grown on substrates made of vineyard wastes during different time periods between 30 and 60 days, varying the inoculum volume (5-7

Table 4 The effects of initial pH and temperature upon mushroom fruit body formation on white poplar and beech composts

Initial pH (pH units)	Initial temperature (t^0)	Final weight of the fresh mushroom fruit bodies (g/ kg substratum)		
		G. lucidum	*L. edodes*	*P. ostreatus*
4.5	18	17 ± 0.23	191 ± 0.10	180 ± 0.02
5.0	21	193 ± 0.15	203 ± 0.05	297 ± 0.14
5.5	23	198 ± 0.10	195 ± 0.15	351 ± 0.23
6.0	26	181 ± 0.12	179 ± 0.12	280 ± 0.03
6.5	29	173 ± 0.09	105 ± 0.23	257 ± 0.15

Table 5 The effect of inoculum age upon mushroom fruit body formation on white poplar and beech composts

Inoculum age (h)	Final weight of the fresh mushroom fruit bodies (g/kg substratum)		
	G. lucidum	*L. edodes*	*P. ostreatus*
264	123 ± 0.14	128 ± 0.05	135 ± 0.23
240	141 ± 0.10	150 ± 0.28	157 ± 0.17
216	154 ± 0.12	195 ± 0.90	193 ± 0.15
192	155 ± 0.23	221 ± 0.25	215 ± 0.05
168	169 ± 0.37	235 ± 0.78	241 ± 0.07
144	210 ± 0.20	248 ± 0.03	259 ± 0.12
120	230 ± 0.15	253 ± 0.05	264 ± 0.21
96	215 ± 0.09	230 ± 0.15	253 ± 0.10
72	183 ± 0.05	205 ± 0.23	210 ± 0.05

Table 6 The effect of inoculum volume upon mushroom fruit body formation on white poplar and beech composts

Inoculum Volume (v/w)	Final weight of the fresh mushroom fruit bodies (g/kg substratum)		
	G. lucidum	*L. edodes*	*P. ostreatus*
7.0	234 ± 0.12	215 ± 0.20	220 ± 0.05
6.5	245 ± 0.15	248 ± 0.23	251 ± 0.20
6.0	253 ± 0.1	257 ± 0.07	280 ± 0.15
5.5	243 ± 0.12	235 ± 0.03	247 ± 0.07
5.0	255 ± 0.23	215 ± 0.15	235 ± 0.03

v/w). All the experiments were carried out at 25°C and initial pH 5.5. As it is shown in Tables 5 - 6, the inoculum age of 120 h as well as an inoculum volume of 6.0 (v/w) have beneficial effects on the fungal biomass production.

Discussion

From all these fungal species tested, *P. ostreatus* was registered as the fastest mushroom (25 -30 days), then *L. edodes* (35-45 days), and, eventually, *G. lucidum* as the longest mushroom culture (40-50 days). The registered data revealed that the white poplar and beech wood wastes have to be used as substrates for mushroom growing only after some mechanical pretreatments (such as grinding) that could breakdown the whole lignocellulose structure in order to be more susceptible to the fungal enzyme action (Chahal 1994). Due to their high content of carbohydrates and nitrogen, the variants of culture composts supplemented with wheat grains at the ratio 1:10 and rice grains at the ratio 1:5 as well as a water content of 60% were optimal for the fruit body production of *P. ostreatus* and, respectively, *L. edode*s (Ropars et al. 1992). The mushroom culture of *G. lucidum* does not need such supplements (Lamar et al. 1992). So far, lignocellulose biodegradation made by mushroom species of *Ganoderma* genus had been little studied, mostly because of their slow growth, difficulty in culturing as well as little apparent biotechnological potential. Only P. Stamets (1993) reported a few experimental data concerning the cultivation of such fungal species in natural sites and he noticed its slowly growing. In spite of these facts, some strains of *G. lucidum* were grown in our experiments on culture substrates made of wood wastes of white poplar and beech mixed with rye grains at the ratio 1:7 and a water content of 50%. Higher ratio of rye grains might lead to an increase of total dry weight of fruit body, but also could induce the formation of antler branches and smaller fruit bodies than those of the control samples. The final fruit body mushroom production ranged between 15-20 kg relative to 100 kg of compost depending on the specific strains of those three tested fungal species.

Conclusions

From all these fungal species tested in our experiments, *P. ostreatus* was registered as the fastest mushroom culture (25-30 days), then *L. edodes* (35-45 days), and finally, *G. lucidum* as the longest mushroom culture (40-50 days).

The registered data revealed that when the cells were grown in the maltose medium, the fungal biomass production was the highest among the tested variants.

From five nitrogen sources examined, rice bran was the most efficient for mycelia growth and fungal biomass production

Among the various mineral sources examined, K_2HPO_4 yielded good mycelia growth as well as fungal biomass production and for this reason it was as a favourable mineral source.

The inoculum age of 120 h as well as an inoculum volume of 6.0 (v/w) have beneficial effects on the fungal biomass production and the optimal pH and temperature levels for fungal fruit body production were 5.0-5.5 and 21-23° C.

The final fruit body mushroom production ranged between 15-20 kg relative to 100 kg of compost depending on the specific strains of those three tested fungal species.

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