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Research Article

Effect of the lignocellulose substrate type on mycelium growth and biocomposite formation by Ganoderma lucidum GA3P

Galena Angelova[1], Mariya Brazkova[1]✉, Bogdan Goranov[2]

[1] *Department of Biotechnology, Technological Faculty, University of Food Technologies, Plovdiv, Bulgaria*
[2] *Department of Microbiology, Technological Faculty, University of Food Technologies, Plovdiv, Bulgaria*

Abstract
The lignocellulose agricultural wastes, one of the major environmental pollutants, represent an extremely rich resource with high nutritional value, which can be used in the production of value-added products. In the current study the effect of different lignocellulose substrates on the growth rate of *Ganoderma lucidum* GA3P and the formation of mycelium-based bio-composites was determined. The macromorphology and specific mycelial growth rate of the colonies on different media containing various lignocellulosic substrate were studied. The obtained composites were characterized regarding their density of the mycelial growth, apparent density and size. *G. lucidum* GA3P demonstrated high μ_{max} values ranging from 0.267 d^{-1} to 0.558 d^{-1} and low K values indicating that all used media were suitable for cultivation, but when wheat bran was used, the formed mycelium-based bio-composites possessed the best characteristics with highest apparent density recorded (0.39 ± 0.005).

Keywords
lignocellulose, Ganoderma lucidum, mycelium-based bio-composites

Abbreviations
BP – beep presses; HERF – hexane extracted rose flower; MCM – Mushroom complete medium; MR – malt roots; PS – pine sawdust; SDLS – steam distillated lavender straw; WB – wheat bran; WS – wheat straw

✉*Corresponding author: Mariya Brazkova, Technological Faculty, University of Food Technologies, Plovdiv, Bulgaria, tel.: +359 32 603 828; E-mail: mbrazkova@uft-plovdiv.bg*

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Introduction

During the last decade the interest of researchers regarding valorization of waste materials is increasing. Various wastes from agriculture and forestry are been used as substrates for the obtaining of natural bio-materials with multiple applications (Sun et al. 2019). Traditional ideas of material production are now being revolutionized with the introduction of biological agents. The new materials contain a biological component binding the lignocellulose substrates. The focus of the scientific community has now been shifted to the development of new sustainable and biodegradable materials with biological origin mainly because of the growing need of "green" materials leading to pollution reduction. The "growing design" approach refers to growing materials from living organisms to achieve unique material functions and sustainable solutions for design, architecture and industry (Karana et al. 2018).

One of the major pollutants of the environment are the lignocellulose wastes generated from agricultural activities. These wastes possess high nutritional value and have considerable potential for application in renewable energy production or value-added products (Piri et al. 2018). The recovery of lignocellulose residues nowadays represents a good strategy for prevention of fossil fuels depletion and minimization of greenhouse gas emissions. Lignocellulose waste combines three major components in its composition – cellulose (40-50%), hemicellulose (20-30%) and lignin (10-25%) which form complex three-dimensional structures depending on the origin of the lignocellulose biomass. Depending on the source, ash, protein and pectin could also be found in the lignocellulose material (Perez et al. 2002).

Numerous microorganisms are known to be able to use cellulose and hemicellulose as the sole source of carbon and energy for their growth. Lignin degraders are even fewer and only some representatives from the Kingdom Fungi have the ability to fully mineralize lignin. These white and brown rot wood-decaying fungi are part of the *Agaricomycetes* class and their lignocellulose activity plays an important role in the plant waste degradation in nature (Bilal et al. 2017). Various types of lignocellulose wastes are being studied for application in bio-composite formation. The properties of the obtained composites are highly dependent of the type of the used substrate. The selection of substrates suitable for fungal growth and obtaining of mycelium-based composite material is the key step in the development of an applicable technology. These composites are now an attractive alternative to synthetic materials and have numerous possible applications in architecture and design, packaging, construction, etc. One of the interesting scientific studies on mycelium based composites is the development of a technology for building self-growing homes.

The aim of this study is to evaluate the effect of the type of lignocellulose substrate on the mycelium growth of *Ganoderma lucidum* GA3P and the formation of mycelium based bio-composite materials.

Materials and Methods

Microorganism. The microbial strain used in this study was *G. lucidum* GA3P, part of the microbial collection of the Biotechnology Department at University of Food Technologies, Plovdiv, Bulgaria. The strain was maintained on Mushroom complete medium (MCM) with the following composition ($g.dm^{-3}$): Glucose – 20.0; KH_2PO_4 – 0.5; K_2HPO_4 – 1.0; $MgSO_4 \times 7H_2O$ – 0.5; peptone – 2.0; yeast extract – 2.0, supplied by Merck KGaA (Germany). The pH prior to sterilization was adjusted to 6.00 - 6.20. Cultures were incubated at 28 °C for 7 days and stored afterwards at 4 °C.

Substrates and preparation for cultivation. Wheat straw (WS), pine sawdust (PS), wheat bran (WB), malt roots (MR), beet presses (BP), hexane extracted rose flowers (HERF) and steam distillated lavender straw (SDLS) were used as feeding lignocellulose substrates.

For the first stage of the experiment six different agar media, containing the abovementioned lignocellulose substrates were prepared. The composition of these media is shown in Table 1. The substrates were ground, added to 2% agar and sterilized at 121 °C for 30 min.

The second stage of the experiment included the cultivation of the macromycete on single substrates – PS, WS, WB, HERF and SDLS. Prior to their sterilization the substrates were moisturized up to 65-70% using the following solution ($g.dm^{-3}$):

$MgSO_4$ x $7H_2O$ – 0.5; KH_2PO_4 – 0.5; K_2HPO_4 – 1.0; peptone – 2.0; yeast extract – 2.0. The moisture was determined on MAC 50/NH moisture analyzer (RADWAG, Poland). Substrates were mixed well and 20 g of each was transferred into Petri dishes (d=10 cm) and sterilized at 121 °C for 45 min. These were used afterwards for determination of the mycelium characteristics and the specific mycelium growth rate.

Table 1. Media for determination of mycelium growth rates

	PS	WB	WS	HERF g.dm^{-3}	SDLS	$CaCO_3$
Medium 1	40	-	-	-	-	2
Medium 2	20	20	-	-	-	2
Medium 3	-	-	40	-	-	2
Medium 4	20	-	20	-	-	2
Medium 5	-	-	-	40	-	2
Medium 6	-	-	-	-	40	2

In the third stage fourteen different combinations (Table 2) of the lignocellulose substrates were used for solid-state cultivation of *G. lucidum* GA3P and for formation of mycelium-based bio-composites. The substrates were previously ground to size of 1-5mm.

Table 2. Combinations of lignocellulose substrates

	WS	PS	WB	HERF %	SDLS	MR	BP
B1	100	0	0	0	0	0	0
B2	0	100	0	0	0	0	0
B3	0	0	100	0	0	0	0
B4	0	0	0	100	0	0	0
B5	0	0	0	0	100	0	0
B6	80	20	0	0	0	0	0
B7	70	30	0	0	0	0	0
B8	60	40	0	0	0	0	0
B9	50	50	0	0	0	0	0
B10	40	40	10	0	0	10	0
B11	30	50	20	0	0	0	0
B12	30	20	40	0	0	10	0
B13	20	20	30	0	0	20	10
B14	10	60	10	0	0	20	0

Prior to sterilization the lignocellulose substrates were moisturized up to 65-70% using the following solution (g.dm^{-3}): $MgSO_4$ x $7H_2O$ – 0.5; KH_2PO_4 – 0.5; K_2HPO_4 – 1.0; peptone – 2.0; yeast extract – 2.0. The moisture was determined on MAC 50/NH moisture analyzer (RADWAG, Poland). Substrates were mixed well and 20 g of each combination transferred into Petri dishes (d=10 cm) and sterilized at 121 °C for 45 min.

Inoculation of the substrates and mycelium growth. A well developed, 7-day culture of *G. lucidum* GA3P was used for the inoculation of nutrient media in all stages of this experiment. For the first and second stage the inoculation was performed with an agar disk (d=10 mm) of the culture which was placed in the center of the Petri dish. The cultivation was carried out at 28 °C for 14 days and in the absence of light. The basic morphological characteristics of the culture were evaluated by taking into account the surface, shape, density, colour and growth of the colony. For the obtaining of mycelium-based bio-composites 5% vegetative inoculum was used and homogenized well with the substrates under sterile conditions.

Determination of mycelium growth rate. The diameter of the formed colonies was measured every day during cultivation and the mycelium growth rate was determined by the equation (1):

$$\frac{dD}{d\tau} = \mu_{max}D - KD^2 \rightarrow \mu D - \frac{\mu}{D_m}D^2 \rightarrow \mu_{max}(1 - \frac{D}{D_m})D$$

$$K = \frac{\mu_{max}}{D_m} \qquad (1)$$

where μ_{max} is the maximum growth rate, D and D_{max} are current and maximum colony diameter and K is the growth retardation constant.

Obtaining of mycelium-based bio-composites. For the obtaining of mycelium-based bio-composites with *G. lucidum* GA3P a two-step cultivation process was applied. In the first step *G. lucidum* GA3P was incubated in Petri dishes at 28 °C and in the absence of light until the formation of white mycelium coating on the substrate surface. At the second stage the formed composite materials were transferred aseptically in a climatic chamber at 28 °C and 95% humidity until full coverage of the composite surface with mycelium. After the end of the cultivation the mycelium-based bio-composites were placed in a heating chamber where the fungal

strain was heat-killed at 60°C for 8 h. The obtained composites were characterized regarding their mycelial growth, apparent density and size. The apparent density of the composites was determined using the following equation (2):

$$\rho_a = \frac{m_d}{V_d} \qquad (2)$$

where ρ_a is apparent density, g.dm^{-3}; m_d – weight of the sample, g; V_d – volume of the sample, dm^3.

The dimensions for the volume determination were measured using caliper with a precision of 0.01 mm. Each dimension was measured three times and the average value was used.

Results and Discussion

The lignocellulose substrates used in the present study were chosen because of their high worldwide distribution. In Bulgaria large quantities are generated as a by-product of agricultural and industrial processes. These substrates are a promising source of cellulose and lignocellulose and their content in the studied substrates is an important factor that affects the mycelium growth of the basidiomycetes, the speed of colonization of the substrates and the physic-mechanical properties of the obtained mycelium-based bio-composite. The lignocellulose, together with the non-cellulose polysaccharides are the major carbon source for the vegetative growth of the mushrooms. The vegetative growth is also associated with intense colonization and lignocellulose degradation due to the many enzyme activities possessed (endoglucanase, β-glucanase, xylanase, laccase etc.) (Haneef et al., 2017). On the other hand, the presence of lignin, cellulose and ligno-cellulose fibres enhance the structure of the obtained bio-composites due to the crystalline structure of the cellulose and the protective role of lignin in the lingo-cellulose matrix.

The growing pollution and the need of novel biodegradable materials let to the development of the so-called self-grown materials which attract major research interest in the past few years. The great number of possible matrixes and biological components that could be employed in the development of such materials gives numerous research possibilities. *G. lucidum* was used as biological component in the mycelium bio-composite formation by several authors where cotton stalk, oat straw, birch sawdust and cellulose were used as matrixes (Haneef et al. 2017; Liu et al. 2019; Tacer-Caba et al. 2020). However, the number of papers dealing with the growth kinetics of *G. lucidum* on lignocellulose substrates for bio-composite formation is still scarce.

An object of the current study was the macromycete fungi *G. lucidum* GA3P. As a mushroom representative this strain has specific morphogenesis and is able to form either fruiting bodies called basidiocarp, or vegetative mycelium which has white to creamy colour and velvety texture of crossed hyphae. Precisely that type of growth and the solid-state cultivation of the mushroom on agricultural wastes were of interest in this investigation. In the first stage of the experiment *G. lucidum* was cultivated on six agar media, containing lignocellulose substrates as a source of carbon and energy. The evaluation of the development rate was made by observation of the macromorphology of the colonies formed, which are depicted on Fig. 1.

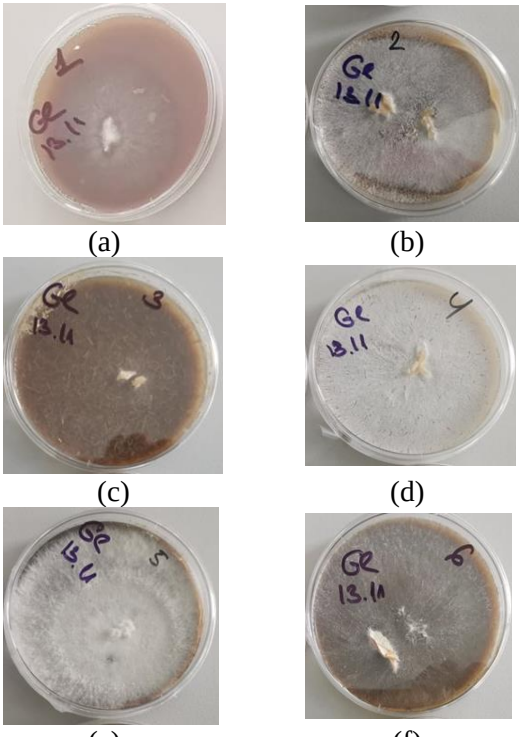

Figure 1. Morphology of *G. lucidum* colonies after 14-day cultivation on Medium 1(a), Medium 2(b), Medium 3(c), Medium 4(d), Medium 5(e) and Medium 6(f)

The specific mycelium growth rate could be used as criteria for the cultivation of basidiomycetes for the obtaining of bio-composites considering the fact that the fast-growing strains possess the ability to better colonize the substrates and thus have bigger influence over the properties of the mycelium-based bio-composite. The high specific mycelium growth rate is not always in correspondence with sustainable bio-composite formation. The basic morphological characteristics and the specific mycelium growth rate for the used lignocellulose substrates are presented in Table 3.

Table 3. Colonies morphology and specific mycelium growth rate of *G. lucidum* GA3P

Medium	Mycelium characteristics				Specific mycelium growth rate			
	Surface	Density	Growth	Color	μ_{max}, d^{-1}	D_m, mm	K, $mm.d^{-1}$	R^2
PS	Loose	Low	Poor	White	0.513	94	0.0055	0.9638
WB	Velvety	High	Intense	Yellow	0.551	93	0.0059	0.9782
WS	Loose	Low	Poor	White	0.267	72	0.0022	0.9582
HERF	Loose	Moderate	Moderate	Pigmented	0.558	92	0.0061	0.9952
SDLS	Loose	Moderate	Moderate	Pigmented	0.362	98	0.0037	0.9855

Basidiomycetes demonstrate two types of mycelial surface on lignocellulose substrates - velvety or loose. The second one is associated with lower mycelial density and poor growth. *G. lucidum* demonstrated high μ_{max} values ranging from 0.267 d^{-1} to 0.558 d^{-1} and low K values indicating that all used media were suitable for cultivation. When grown on lignocellulose substrates with high content of complex polysaccharides poor to moderate growth was observed and the time required for the substrate colonization was be longer.

When grown on PS and WS as the sole source of carbon and energy scarce or no growth was observed. The pine sawdust composition varies with different tree species and their geographic location. In general, the holocellulose concentration is the highest, reaching up to 70% followed by the lignin with 20-25% concentration (Chávez-Rosales et al. 2021). Sawdust from different deciduous trees was used as substrate for the growth and fruiting body formation of *G. lucidum* species where Roy et al. (2015) observed poor mycelium formation despite the fact that the sawdust was used in combination with rice or wheat bran as additional, easily degradable supplements of nutrition elements (Roy et al. 2015). Wheat straw composition could be summarized to cellulose (28-39%), hemicelluloses (23-24%) and lignin (16-25%), with lower contents of ash and protein (Carvalheiro et al. 2009). The high contents of cellulose, hemi- and holocellulose and lignin was the main reason for the scarce growth of *G. lucidum* on PS and the absence of growth on WS.

The vegetative development of the employed fungal strain is highly dependent on the content of lignocellulose compounds in the substrates. In this regard the poor growth on SDLS was expected since this substrate has high cellulose and lignin concentrations. The best growth was observed on HERF which has lower cellulose and lignin concentrations in comparison with SDLS (Angelova et al. 2021) which means that the components were more easily accessible to the fungi. When grown on HERF and SDLS colonies formed yellow to brown exudate on their surface. Those brownish spots are commonly reported and accepted as a result of the depolymerization of polysaccharides and the melanin production in the early stages of the lignocellulose substrate degradation (Sun et al. 2019; Ridzqo et al. 2020).

The process of developing mycelium-based bio-composites is based on a two-stage solid-state cultivation of *G. lucidum.* During stage I the cultivation took place in Petri dishes where the initial colonization of the substrate occurred. The composite mass was formed by agglutination between the lignocellulose substrate and the mycelium of the mushroom. This composite mass took the form of the Petri dish and could be shaped in any form that it was grown in. The aim of the stage II cultivation was the stimulation of the mycelial growth and the formation of air mycelium and also determination of the suitability of the

chosen lignocellulose substrates for the development of mycelium-based bio-composites. As the mycelium grows the bonds in the lignin and the cellulose are partially degraded and new bonds between the mycelium and the substrates are formed. The mushroom secretes polysaccharides, hydrophobines and other metabolites with contribute to the formation of thick composite material.

Wheat straw and pine sawdust were not the best choices for substrates for the obtaining of mycelium-based bio-composite with *G. lucidum*.

The data (Table 4) suggests that when WB was used as lignocellulose substrate the obtained bio-composite materials possessed the best characteristics. Wheat bran is the main by-product of white flour production and due its high nutritional value is often used as an additive in animal feed. It consists mainly of non-starch dietary fibers such as cellulose and arabinoxylans (Weiser et al. 2020).

Table 4. Characterization of the mycelium growth and the formation of mycelium-based bio-composites with *Ganoderma lucidum* cultivated in different lignocellulose substrates

Medium	Density of the air mycelium*	Stage I, days	Stage II, days	Apparent density, $g.dm^{-3}$**
B1	++	25	na	na
B2	++	25	na	na
B3	+++	25	35	0.19 ± 0.003
B4	++	28	38	0.39 ± 0.005
B5	++	28	38	0.31 ± 0.004
B6	+	na	na	na
B7	+	na	na	na
B8	+	na	na	na
B9	+	na	na	na
B10	+	na	na	na
B11	+	na	na	na
B12	++	30	40	0.23 ± 0.003
B13	++	30	40	0.20 ± 0.003
B14	+	na	na	na

* Rate of mycelium density "+" – scarce surface growth not leading to composite formation, "++" – there is surface growth and slight agglutination of the substrate particles, "+++" there is surface mycelium growth but no mycelium layer is formed, "++++" there is surface growth and mycelium layer formed

** All values are expressed as mean ± SD of triplicate measurements

Due to its composition it was easy for *G.lucidum* to grow and form mycelium net wrapping it. The other two media with potential for bio-composite formation were B12 and 13 where the ratio of components (PS, WS and WB) was in favor of the WB thus explaining the surface growth and the slight agglutination of the substrate particles. *G. lucidum* also developed mycelium bio-composites when grown on HERF and SDLS, with the highest apparent density recorded for the HERF substrate (0.39-0.005 $g.dm^{-3}$), where the strain had the highest specific mycelium growth rate (0.558 d^{-1}). The obtained materials were brittle and had yellow to brown color probably due to Maillard reactions between the sugars and proteins in the substrate and the fungi during the drying of the composites or might be due to lowered water content, as hypothesized by Apples et al. (2019). The structure of the composites was easily crushed and very unstable (Fig. 2-b,c). It was obvious that the structures obtained from PS and WS were very unstable and even slight pressure leads to the collapse of the composite material (Fig. 2-d,e). The possible reason for this is the assumption that *G. lucidum* grows only on the surface of the substrate without entering the depth of the material. PS and WS consist mainly of cellulose and lignin which makes them difficult to decompose with lignin-

degrading enzymes. When grown on PS *G. lucidum* formed small tubular hyphae with low density on small surface areas of the substrate and did not penetrate in depth. These observations are supported by the findings of Alves et al. (2019). The lack of proper colonization of the substrates could be explained with the low availability of the nutrients in the substrates on one hand and the insufficient lignin-degrading enzyme activities in *G. lucidum* on the other.

The development of a stable structure of the mycelium-based bio-composites requires thorough optimization of the parameters for cultivation and drying, as well as tuning of the material. The choice of the suitable substrate for the biological agent is only the first step of a successful technology. The future applications of those materials are the starting point for the tuning of the physico-mechanical properties.

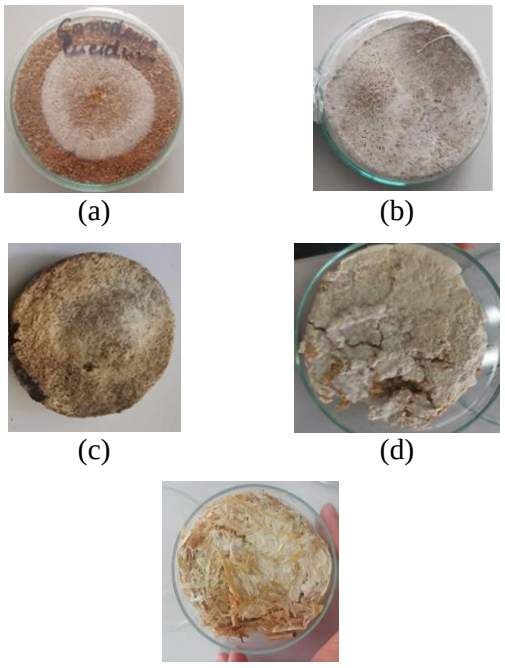

Figure. 2. Mycelium-based bio-composites formed by *G.lucidum* cultivated on WB (a), HERF (b), SDLS (c), PS (d) and WS (e)

Conclusions

The fungus *G. lucidum*, as all fungi, has specific morphogenesis and is able to produce numerous bioactive compounds. Its vegetative growth is associated with intense colonization and substrate decay due to the strain's enzyme activities. The Basidiomycete demonstrated two types of mycelium surface when grown on lignocellulose substrates – velvety and loose. The values for the specific growth rate of *G. lucidum* indicate that all of the investigated substrates used are suitable for cultivation of the strain. The mycelium-based bio-composite obtained with WB as substrate has the best mechanical properties in comparison with the ones produced with the other lignocellulose substrates. Further experiments regarding the optimization of the process for bio-composite formation are needed.

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