# Growth Characteristics and Ergosterol Content of *Grifola frondosa* in Various Solid-state Substrates

**A. Gregori,[a] M. Švagelj,[b] D. Voglar,[b] and M. Berovic[b,*]**

[a]Institute for Natural Science, Ul. Bratov Ucakar 108, SI-1000 Ljubljana

[b]Department of Chemical, Biochemical and Environmental Engineering, Faculty of Chemistry & Chem. Technology, University of Ljubljana, Vecna pot 113, 1000 Ljubljana, Slovenia

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Growth characteristics of medicinal mushroom *Grifola frondosa* mycelia were studied in solid-state cultivation on various solid-state substrates in different setups. The mycelial growth rate was determined in racing tubes by the measurement of ergosterol content. The fastest growth rate of 3.76 mm $d^{-1}$ and the highest biomass amount of 54 mg $g^{-1}$ were achieved. The results were scaled-up in a horizontal stirred bioreactor, where higher amounts of biomass (53.2 mg $g^{-1}$ in 42 days) were achieved. These results represent an available platform for large-scale production of medicinal fungi biomass in bioreactors.

*Key words:*
*Grifola frondosa*, biomass, racing tubes, ergosterol, horizontal stirred bioreactor

## Introduction

*Grifola frondosa* (Dicks: Fr) S.F. Gray is a white rot basidiomycete, belonging to *Basidiomycotina, Homobasidiomycetes, Aphyllophoromycetideae, Polyporales,* Family *Meripilaceae*[1]. The mushroom (Figure 1) is known under several English names (Dancing mushroom, Cloud mushroom, Hen-of-the-woods, the Dancing Butterfly mushroom, Sheep's head), in Japan as Maitake, and in China as Hui Shu Hua. Fresh and dried *G. frondosa* fruit bodies, which are commonly available in food markets of Asia, especially of Japan, China and Korea, have also become available in other countries, such as the USA, Canada, and EU. Numerous nutraceutical preparations based on *G. frondosa* powdered fruit bodies, dried extracts or purified polysaccharides are emerging in different forms – as capsules, tablets, as well as additives to food formulations.

*Grifola frondosa* mycelium and its polysaccharides can be produced in liquid media[2] as well as solid-state cultivation on sunflower seed huls[3] and different grain types[3,4], while fruiting bodies are usually cultivated on broadleaf sawdust-based substrates with addition of different supplements, such as wheat bran, crushed cord seeds[5], olive oil press cakes[6] and others. Commercially, polypropylene bags or bottles are used as substrate containers from which fruiting bodies emerge.

Fruiting bodies are large, soft-fleshed, edible, and consist of approximately 86–91 % moisture and 9–14 % dry matter, of which carbohydrates represent 59–60 %, crude protein 21–22 %, crude fiber 10 %, crude fat 3–4 %, and ash 5–7 %[1, 7]. Fruiting bodies also contain ergo sterol, assorted vitamins and minerals[8].

*Grifola frondosa* fruit bodies often occur as a heavy mass at the base of stumps and on the roots of dead or dying deciduous hardwoods, such as *Quercus*, *Ulmus*, *Acer*, *Nyssa*, *Larix* and *Fagus*; sporadically also on *Castanea, Prunus, Pseudotsuga* and *Pinus*. The optimal growing conditions exist within a limited range of temperature, moisture, humidity, and other environmental factors. Parts of northeastern Japan are especially hospitable for this mushroom, although it can also be found in the northern temperate forests of Asia, Europe, and eastern North America[9].

Beside the references cited above, other broadleaf sawdust types could have a potential use for *G. frondosa* biomass and fruiting bodies cultivation. In this article, *G. frondosa* mycelium growth traits on selected substrates composed of beech, Norwegian spruce, European larch and common grape vine sawdust were conducted, and fungal biomass were determined. This work represents the investigation of presently unexploited raw materials for *G. frondosa* biomass and fruiting bodies production. Substrate compositions with the best characteristics for *G. frondosa* growth were evaluated, selected, and applied in a pilot solid-state horizontal stirred bioreactor.

*Address all correspondence to Marin Berovic: Tel: + 386 1 479 8573; marin.berovic@fkkt.uni-lj.si


Fig. 1 – *Fruiting body of G. frondosa in artificial cultivation on beech sawdust supplemented with wheat bran*

## Materials and methods

### Microorganism and seed culture preparation

*Grifola frondosa* (Dicks: Fr) S.F. Gray strain GF3, original strain from Slovenian forests used in this study, was isolated and is deposited at the Fungal bank of the Biotechnical Faculty, Department of Wood Science and Technology, University of Ljubljana, Slovenia. The culture was maintained at 28 °C on potato dextrose agar – PDA (Difco, USA), and transferred every two weeks onto freshly prepared media. Liquid inoculum for PDA plate inoculation was prepared by mixing a two-week-old PDA plate overgrown with *G. frondosa* mycelium in 200 mL sterilized deionized water in a S700 blender (Waring products, Calhoun, GA, USA), three times, for 20 seconds.

The seed culture for the pilot solid-state reactor was prepared by transferring 50 mL of the liquid inoculum to 450 mL sterilized liquid growth medium composed of glucose (30 g $L^{-1}$), yeast extract (6 g $L^{-1}$), peptone (2 g $L^{-1}$), $MgSO_4 \cdot 7H_2O$ (0.5 g $L^{-1}$), $K_2HPO_4 \cdot 3H_2O$ (0.66 g $L^{-1}$), $MnSO_4 \cdot 1H_2O$ (0.14 g $L^{-1}$), pH = 5.5 in a 1000-mL Erlenmeyer flask. The seed culture was incubated for two weeks as a shaken culture at 28 °C and 150 rpm.

### Mycelium growth *in vitro*

Twenty-one petri dishes ($H$ =13 mm, $r$ = 43 mm) containing 35 mL of sterilized PDA were inoculated in the center of the PDA plate with 10 μL of liquid inoculum. Incubation was performed at 28 °C. Mycelium growth was followed with digital images analysis using the Motic Images Plus software (Motic China Group LTD) for 21 days, and expressed as radius of the circular growth zone. Once the mycelium overgrew the petri plate, it was carefully collected from the media surface, dried, weighed, and analysed for ergo sterol content.

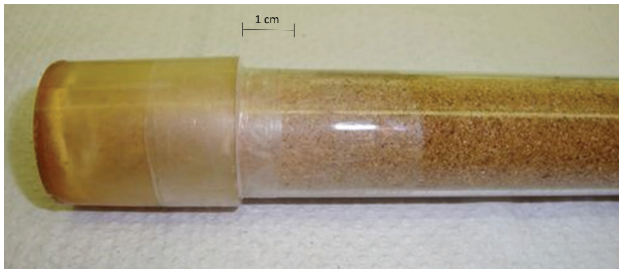


Fig. 2 – *Racing tubes showing solid-state cultivation on beech sawdust substrate. Mycelial growth is visible with the naked eye.*

### Substrates for solid-state cultivation

Substrates were prepared from 76 % dry weight beech (*Fagus sylvatica*), Norwegian spruce (*Picea abies*), European larch (*Larix deciduas*) or common grapevine (*Vitis vinifera*) sawdust (particle sizes from 0.125 mm – 0.5 mm) with the addition of wheat bran (20 % dry weight), $CaCO_3$ (2 % dry weight), and olive oil (2 % dry weight). Substrates water content was adjusted to 65 %.

### Mycelium growth in racing tubes

Growth rate measurements, biomass production, and ergosterol content were studied in solid-state bioprocessing conditions using "racing tubes". The method for growth rate measurements in the samples was developed. Glass tubes 175×25 mm, (Figure 2) were filled with 28 g of substrate (substrate density of 0.35 g $mL^{-1}$), closed on both sides with a plastic cap, and sterilized for 45 minutes at $T$ = 121 °C; $p = 1.2 \cdot 10^5$ Pa. After cooling, the substrates were inoculated on one side of the tube with a 16-mm disc from a two-week-old PDA culture, and wrapped in parafilm to prevent moisture loss. The inoculated substrate in the racing tubes was incubated at 28 °C and mycelium growth was monitored periodically using a stereo microscope joined with a precision measuring tool. The length of the linear growth zone of mycelia in the racing tubes in different time-points represents the growth rate. All experiments were performed in at least triplicate.

### Mycelium growth in glass jar bioreactors

Cylindrical glass jars of 700 mL volume were used as containers for fungal biomass cultivation. They were filled with 400 mL of substrate, and closed with a cotton filter lid for air exchange. The substrates were sterilized for 45 min at $T$ = 121 °C; $p = 1.2 \cdot 10^5$ Pa, inoculated with 5 mL of liquid inoculum, thoroughly mixed and incubated at 28 °C for 7 days. Samples were obtained every three days, and the ergosterol and moisture content determined. All experiments were performed in triplicate.

Electron micrographs of *G. frondosa* mycelium on solid-state substrates were performed with a Field-emission Scanning Electron Microscope at an accelerating voltage of 1 kV (Carl Zeiss Supra 35 VP).

### Mycelium growth in horizontal stirred tank bioreactor

The best substrate formula (and perhaps time of solid-state cultivation) from the previous step – the glass jar bioreactors, were scaled up in an *ad hoc* 15-L horizontal stirred tank bioreactor. Details of the construction of this device are shown in Figure 3. The reactor was filled to two-thirds depth with substrate (3500 g of beech sawdust substrate), sterilised *in situ* for 120 minutes ($T$ = 121 °C; $p$ = $1.2 \cdot 10^5$ Pa), and cooled to the inoculation temperature. Substrate was inoculated with 500 mL of seed culture, and incubated at 28 °C. Periodical mixing (80 rpm) by horizontal blades attached to a central shaft, for two minutes/day, and aeration over a hollow shaft of the impeller (5 L $h^{-1}$ air flow) were applied. The same principles of process optimization as inoculation, sterilization, mixing, aeration, temperature, and humidity were applied as in our previous works[6,10].

### Ergosterol and biomass determination

Ergosterol was determined applying a modified method described by Gregori (2014)[11]. In brief, 10 mg of dry fungal biomass obtained from three petri dish PDA plates were extracted for 90 minutes with 1 mL of a solution of 10 % (w/v) polyvinylpyrrolidone (Sigma, Germany) in 96 % ethanol, at 4 °C on a rotary shaker at 200 rpm. The resulting extract was centrifuged (10000 rpm, 15 min, $T$ = 4 °C), and filtered through a 0.45-μm pore size filter. The filtrate (100 μL) was injected into a HPLC system (Varian, USA) with 1:1 (v/v) methanol:acetonitrile (Sigma, Germany) mobile phase at 1 mL $min^{-1}$ flow rate on a Synergi MAX-RP C12 column (Phenomenex, USA). Ergosterol was identified using standard retention time and specific peak between 260 and 300 nm obtained by calibration curve preparation using purified ergosterol standard (Sigma, Germany). For ergosterol determination in solid-state cultivation substrates, a 2-g sample was extracted with 20 mL of the same extraction mixture and subjected to the same procedure[12].

### Moisture content determination

The moisture content of substrate samples (grams) was determined with a HR83 Moisture Analyzer (Mettler-Toledo, Switzerland).

### Statistics

All of the cultivation experiments were performed at least in three runs. The results were analyzed for statistical significance by one-way analysis of variance (ANOVA) test using the Statistical Package of the Social Science (SPSS) version 11.0 (SPSS Inc., Chicago, IL, USA). Statistical significance of the test effects was evaluated at $p < 0.05$. Data were expressed as mean ± SD.

## Results and discussion

### Mycelium growth *in vitro* and ergosterol content determination

Ergosterol is a specific component of fungal cell membranes[12]. With ergosterol being in correlation to fungal biomass quantity, its determination

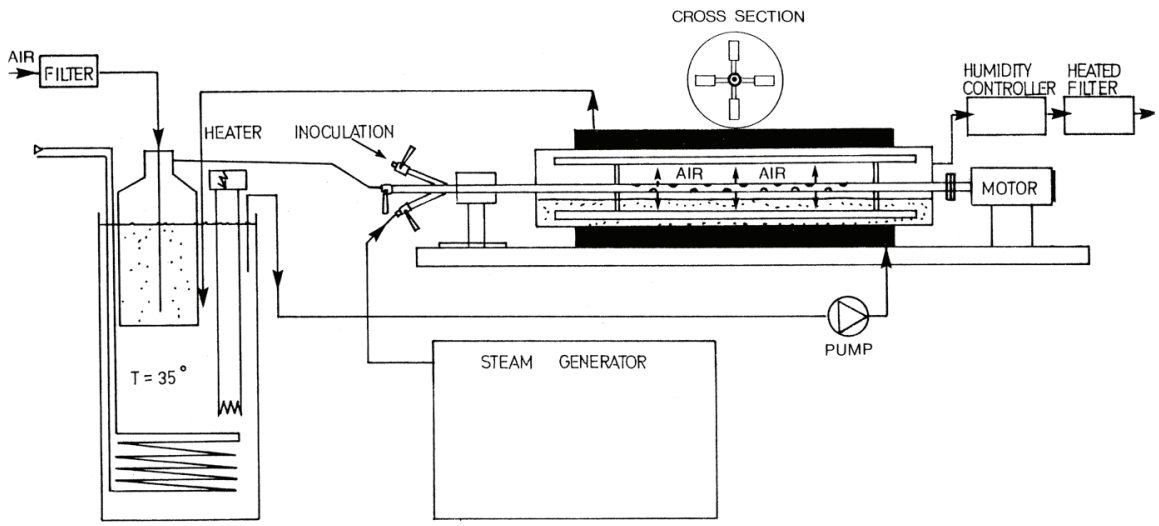


Fig. 3 – *Scheme of horizontal stirred tank bioreactor – according to Berovic and Ostroveršnik (1997)*

can serve as an indicator for fungal growth rate. Figure 4 shows the mycelium growth until day 21 when it reached the borders of the petri plate in all experimental units.

Regarding the ergosterol content in dry biomass, it was found that it was constant throughout the 21 days of the vegetative mycelium growth. The average content of ergosterol in the dried mycelium from the surface of PDA was 5.80 mg $g^{-1}$± 0.04 mg $g^{-1}$. This value was used as reference to calculate the biomass content in the solid-state fermented substrates.

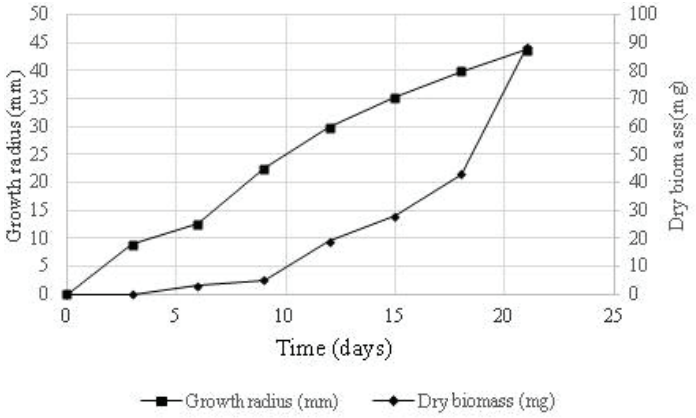


Fig. 4 – *Mycelium growth in vitro. The growth radius and total dry biomass of G. frondosa mycelium on PDA medium are presented.*

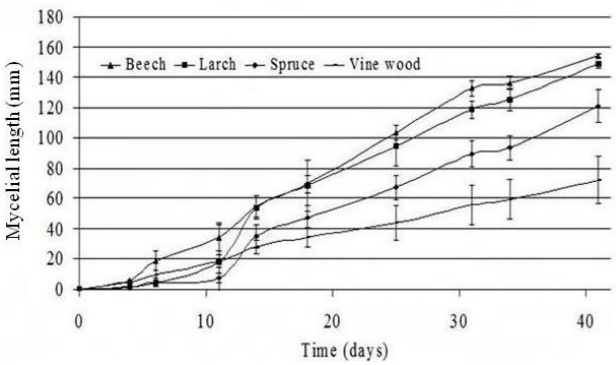


Fig. 5 – *G. frondosa mycelium growth in racing tubes. The growth in millimeters (■) and the biomass dry weight (♦) are presented for different substrates formulations.*

## Mycelium growth in racing tubes

The growth rate of *G. frondosa* on different solid-state bioprocessed fermented substrates using the racing tubes test is shown in Figure 5. The highest growth rate occurred on beech sawdust-based substrate (3.76 mm $d^{-1}$), followed by larch- (3.61 mm $d^{-1}$), spruce- (2.95 mm $d^{-1}$), and common vine (1.76 mm $d^{-1}$) based substrates. Larch- and spruce-based substrates presented a long lag phase that lasted about 12 days, while beech and common vine substrate had no noticeable lag phase.

## Mycelium growth in glass jars

The mycelium growth rate of *G. frondosa* in solid-state bioprocessed substrates in glass jar bioreactors is shown in Figure 6. The highest dry biomass yield of 54 mg $g^{-1}$ solid-state bioprocessed substrate was obtained at day 47 on the beech sawdust-based substrate. Next, were vine (49 mg $g^{-1}$), spruce (40 mg $g^{-1}$) and larch sawdust (30 mg $g^{-1}$) based substrate. Comparing the results from the race tubes and glass jar bioreactors, it was concluded that substrates composed of beech sawdust were more favorable for mycelial growth compared to other substrates. This is well in accordance with the fact that *G. frondosa* in natural habitats occurs mostly on beech wood[8]. The results for growth rates on substrates composed of spruce sawdust are comparable in terms of relative growth rate and biomass accumulation. The growth rate on spruce sawdust substrate was 78 % higher, while in this case the accumulated biomass was only 74 % of that obtained on beech sawdust substrate.

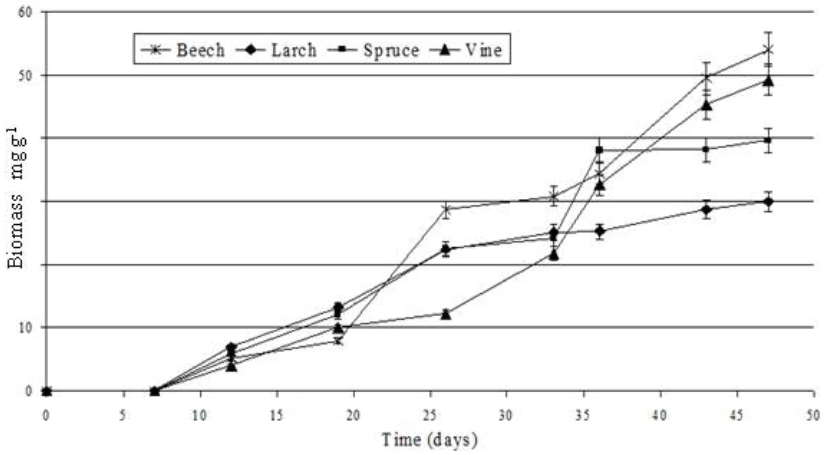


Fig. 6 – *Mycelium growth in glass jar fermenters. Results are expressed as mg of dry biomass per g of solid-state fermented substrate. Main components of substrates are beech sawdust (▲), larch sawdust (■), spruce sawdust (♦) and vine sawdust (—).*

The growth rate on larch sawdust-based substrate was 96 % of the growth on beech substrate, while the amount of biomass was only 56 % of the previous one. This was also visible when comparing the density of mycelia in racing tubes and glass jars. The mycelium was markedly less dense. Interestingly, the data for vine sawdust substrate was more favorable in terms of biomass accumulation, which achieved 90 % of biomass accumulation on beech sawdust substrate, but showed lower growth rate (56 % of the growth rate of beech sawdust substrate).

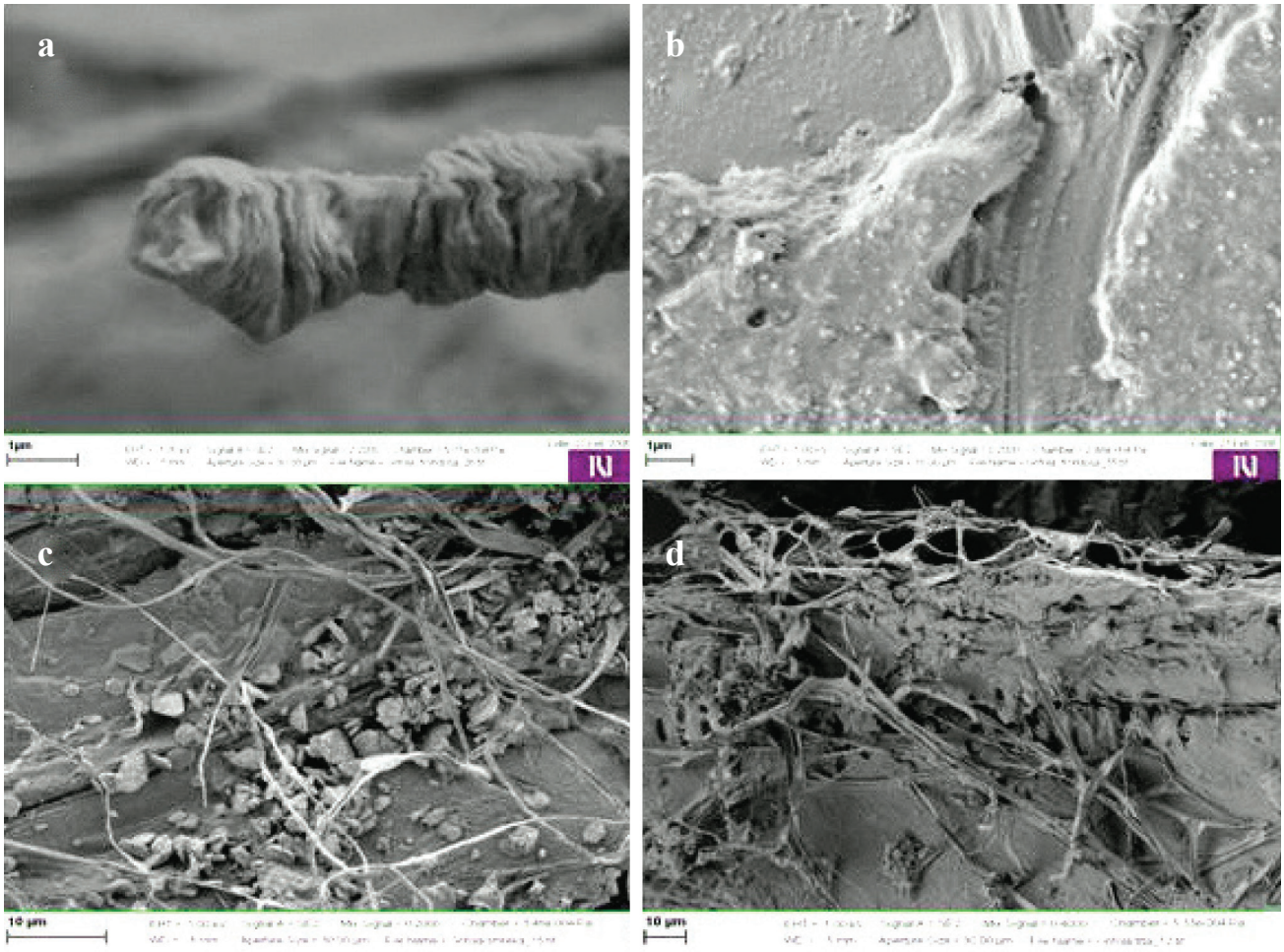


Fig. 7 – *Mycelium from horizontal stirred tank bioreactor. (a) Fungal polysaccharide on the tip of young hyphae. (b) Sticky anchorages of hyphae tips on the cellulose matrix. (c) Mycelium growth on spruce sawdust (d) Vine sawdust-based substrate in the horizontal stirred bioreactor.*

### Mycelium growth in the horizontal stirred tank bioreactor

After the lag phase, mycelium grew concentrically on the surface of the solid particles and coaxially from the perforated shaft of impeller towards the walls of the bioreactor (Figure 3). During growth, the polysaccharides were secreted at the tips of young hyphae (Figure 7a). These molecules were used as sticky materials to produce the anchorage points for the movement of young hyphae, as well as transport medium for secretion of lignocellulolytic enzymes and a protective agent (Figure 7b).

From our results, it was found that growth in the horizontal stirred tank reactor was closely parallel with the growth in the glass jar fermenter achieving the most comparable amount of biomass (53.2 mg $g^{-1}$) in only 42 days. The quicker growth rate can be attributed to aeration, as the glass jar fermenter only had passive ventilation. The total biomass produced in the solid-state fermenter after 42 days of incubation was 65 g dry weight.

## Conclusions

Various substrates based on lignocellulosic sawdust particles from beech, Norwegian spruce, European larch and grapevine (sizes from 0.125 mm – 0.5 mm) were tested. The average ergosterol content in fungal biomass in measured samples was 5.80 mg $g^{-1}$ ± 0.04 mg $g^{-1}$. The growth rates and biomass accumulation were different on each of the prepared solid substrates. As expected, the best results were obtained on beech sawdust substrate (3.76 mm $d^{-1}$ and 54 mg $g^{-1}$). The remarkable differences were indicated on substrates composed of larch and vine sawdust substrates. The former was better in terms of growth rate and the latter in biomass production.

The results from the cultivation process in the horizontal stirred bioreactor, in terms of biomass per amount of solid substrate, compared at the same amount of biomass, indicated a faster cultivation process in aerated bioreactor.

The growth area increased linearly (at 10.55 ± 0.02 SD mm $d^{-1}$), while the biomass increased exponentially. Final dry biomass achieved at 21 days of incubation was 88.3 ± 0. 028 SD mg.

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