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Investigations of mycelium growth and fruit body development of different strains of the beech mushroom Shimeji (*Hypsizygus marmoreus* Bull.: Fries) Singer

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ABSTRACT: The morphological and physiological characteristics of four strains *of Hypsizygus marmoreus* (Bull.:Fries) Singer, an edible, medicinally important mushroom were studied on beechwood substrate, agar and liquid media. The hyphae were found to have clamps and formed arthroconidia, chlamydospores and crystals. The surface growth rate of the mycelium depended on the composition of the medium used and was up to 4.0 mm/day. No growth was observed at 6 and at 37°C. The optimum temperature for mycelium growth was between 23 and 27°C for Japanese strains and between 18 and 23°C for the European strain. The optimum water content of the substrate is 55 to 66%. The optimum pH for growth of mycelium was around 6.1. The strains studied differed in their production of CO_2 during vegetative growth; CO_2 raising above the detection level at day 3. After 9 days the CO_2 concentration increased fast and growth of mycelium was possible at up to 0.9% CO_2.

1 INTRODUCTION

H. marmoreus is an important edible mushroom and occurs in Asia, Europe and North America (Stamets 1993). It was recorded for the first time in China and described briefly with illustrations including cultural characters (Yang et al. 1992). *Hypsizygus* species are cultivated by North American and European growers for flavour and texture characteristics. The total world production of Shimeji mushroom under commercial cultivation in various individual countries in 1994 was 54.8 x 1000 metric tons. It is cultivated mainly in Japan and the production between 1990 and 1994 increased with 140 % (Chang 1999, Royse 1996).

Recently, this mushroom has been attributed to have anticancer properties (Ikekawa 1995, Saitoh et al. 1997, Stamets 1993, Wasser & Weis 1999). The chemical composition of the fruiting bodies and the sugar and amino acid components of the aqueous extract were analysed. The polysaccharide, ß-(1-3)-D-glucan, isolated from this mushroom showed high antitumor activity. Its water solubility was greater than that of other ß-(1-3)-D-glucans isolated from edible mushrooms (Ikekawa 1995). Recently new polyisoprenepolyols were isolated from *H. marmoreus* and tested for their antitumor activities. A fast atom bombardment mass spectrometry was used to analyse the structure of the polyisoprene-polyols (Sawabe et al. 1999).

At this moment it is known that consumption of fruit bodies of *H. marmoreus* may inhibit carcinogenesis and mushroom extract has a radical-trapping activity. Antitumor activity was tested in mice with syngeneic tumours, Lewis lung carcinoma and Sarcoma 180 (Ikekawa 1995, Saitoh et al. 1997). Studies are on-going for a more precise determination of their medicinal properties. The antioxidant effects of *H. marmoreus* are now being studied and the results suggest that oral administration of fruit bodies can induce an antioxidant effect in mouse plasma. This increase of antioxidant activity in the plasma of tumour-bearing mice has a cancer preventive effect. It also suggests that *H. marmoreus* might play a role in decreasing thiobarbituric acid reactive substances (Matsuzawa et al. 1997, Matsuzawa et al. 1998). Thus, *H. marmoreus* has a promising future as a food and as a raw material for the production of

pharmaceuticals. Clearly, there is a need to develop cultivation techniques for this mushroom. The study reported here was carried out to determine the main environmental factors required for a good mycelial growth of different *H. marmoreus* strains.

2 MATERIALS AND METHODS

Four strains of *H. marmoreus* were tested: strain 1 from Mycelia (Belgium) and strains 2, 3 and 4 from Hokuto Corporation (Japan). Stock cultures were maintained in test tubes on malt extract peptone agar (MPA) (g/l): malt extract – 30; peptone – 5 (both from Merck, Germany) and agar-agar –20 (Roth, Germany). The cultures were stored at 4-8°C and subcultured every 6 months. The master cultures were incubated in 90 mm diameter plastic petri dishes (Büchler, Germany) at 25°C on 20 ml MPA.

Vegetative mycelium from different parts of the colony (margin, centre) were examined by SEM (scanning electron microscopy). Inoculum was placed in the centre of petri dishes containing MEA, and 5 to 7 cover glasses (4×4 mm) were placed at a distance of 20 to 50 mm around the point of inoculation. After incubation at 26°C for 3 to 5 days, vegetative mycelium grew over the cover glasses and was transferred to a microscopic slide. The slide was then placed into a sealed glass vessel for fixation with osmium tetroxide vapor (1% solution) for 96 h. After fixation these slides were transferred to an empty petri dish to dry for 72 h. The specimens were examined using a JSM-35C Scanning Electron Microscope (Jeol, Japan) at a magnification of 1.000 to 15.000.

To investigate the morphological and physiological characteristics, the following synthetic, natural, and complex nutrient media were used:

1) Czapek`s medium (CzA) (g/l): Sucrose - 30; $NaNO_3$ - 2.0; KH_2PO4 - 1.0; $MgSO_4*7H_2O$ - 0.5; $FeSO_4*7H_2O$ - 0.01; KCL - 0.5; agar-agar ("Serva ") - 20; pH- 5.8±0.2.
2) Malt extract agar (MEA): Cod CM 59 ("Oxoid"); pH 5.4±0.2.
3) Potato dextrose agar (PDA): Cod CM 139 ("Oxoid"); pH 5.6.
4) Malt yeast pepton agar (MYPA) (g/l): malt extract - 20; yeast extract – 2.0; pepton – 1.0; agar-agar - 20.

The media were sterilized for 30 min. at 121°C then cooled and poured into sterile petri dishes (20 ml) for later use. After 10 days of incubation at 25°C, standard disks (diam of 5 mm) were cut from the margin of a mycelial colony using a sterile stainless steel tube, followed by transfer of the disks to the centre of new petri dishes containing the above-mentioned media using a sterile scalpel. Freshly inoculated petri dishes were incubated at 25°C.

In comparative investigations, two liquid media were used:

5) Liquid malt extract diluted by distilled water, to give a carbohydrate content of 4.0±0.2%.
6) Complex medium (g/l): Glucose – 50; peptone - 5.0; yeast extract - 2.0; $MgSO_4*7H_2O$ - 0.5; $CaCl_2*2H_2O$ - 0.3; KH_2PO_4 -1.0; corn steep liquor - 5 ml; trace metal solution - 10 ml (the composition of the trace metal solution per 500 ml: $MnCl_2*4H_2O$ - 0.2g; $ZnCl_2$ - 0.1g; $FeCl_3*6H_2O$ - 0.42g; $CuSO_4*5H_2O$ - 0.039g).

Medium 6 was used to determine the optimal pH-level. The nutrient solutions were adjusted to different pH-levels with 20% KOH and 1N HCl and dispensed at 20 ml in 100 ml flasks. Media were sterilised for 30 min. at 121°C. Four agar disks (diam 5 mm) with mycelium were used to inoculate the culture flasks for surface cultivation. We used mycelia in the active physiological state in compliance with our current methodology (Weis et al. 1999). For submerged cultivation inoculum shake cultures were made first: mycelium was incubated in 250 ml flasks with 25 ml medium 6 for 5 days at 26°C. On day 6 100 ml of the culture was homogenized in a Warring Commercial Blender for 1.5 min. Aliquots of 10 ml, containing a biomass of 0.9 g/l dry matter, were used as inoculum. The final cultures were done in 75 ml of medium 6 dispensed in 500 ml. The flasks were incubated at 26°C on a rotary shaker at 120 rpm. After incubation on liquid media, culture samples were collected on pored glass funnels, washed with distilled water and dried at 105°C to constant weight.

The influence of the temperature and the humidity of the substrate on the mycelial growth rate was studied in petri dishes filled with 10 g of a dry substrate (80% beechwood sawdust, 20% corn meal). Ten temperatures (6, 16, 18, 22, 23, 25, 26, 27, 30 and 37°C) and seven humidity grades (50, 55, 56, 59, 60, 61 and 66%) were investigated on sterilized substrate. After

autoclaving, the cooled substrates were inoculated with standard mycelium disks (diam 5 mm). Three petri dishes were used for each variant of medium, humidity or temperature studied. Growth of the colonies was monitored every 2 to 3 days in four directions. All figures present the mean data of 12 measurements. When the increase of the colony radius (R2-R1, mm) with time (t2-t1) was linear, the mean of the radial growth rate (VR, mm/day) of each colony was calculated. The dry matter contents of the substrate was determined with a TS Moisture Analyser 30 (Sartorius, Germany), 2 samples of 4 g each for each replicate. Tubes with 20 ml MEA were inoculated with mycelium and grown at 4 and 37°C as controls. After incubation at 4 and 37°C (10 days) the cultures were incubated under optimal conditions at 26°C. The humidity test was done at 26°C.

The production of CO_2 by growth of *Hypsizygus* strains was investigated similar to Grygansky et al. (1999) for *Hericium erinaceus*. Plastic petri dishes of 90 mm diameter were filled to 75% of their volume with 35 to 50 g of the substrate with a moisture content of 63%. One to three holes (diameter of 1 cm) were made in the cover of each petri dish for gas exchange and gas measurements. The substrate was inoculated with three replicates per strain. Petri dishes were closed with Nesko-film and the holes covered with Tesacrepp. Measurements of CO_2 were done every 3 days, with a transportable CO_2 gas analyser (Gas Data PCO_2, DATA Ltd., UK). Measurements of CO_2 in the laboratory room and inoculated petri dishes were used as controls.

Three fast growing strains (strains 2, 3, 4) were tested for fruit body production on blocks of 2 kg of substrate in plastic bags (20 replicates). The formulation of the substrate was the same as that for temperature and humidity investigations. The spawn was alowed to grow for about three months at the optimal temperature for each strain (23-27°C). Bags were opened and brought in a harvest room at 16 to 18°C, light at 500 to 1000 lux and CO_2 at 1000 to 2000 ppm.

3 RESULTS

3.1 *Morphological and cultural characteristics of vegetative mycelium*

The microstructural morphology of the pure culture was studied using scanning electron microscopy. Structures for the asexual dissimination as chlamydospores and arthroconidia were found to be a characteristic feature of the dikaryotic mycelium of this mushroom (Fig. 1). Chlamydospores are numerous, and developed intercalary or apicaly. Terminal chlamydospores dominated. Arthroconidia are connected in simple chains and formed lateral mycelial branches, a condensation of protoplasm was not observed. Crystals of various form and size were observed.

In general, the cultural characteristics of *H. marmoreus* colonies of the different strains were similar. Mycelial growth was slow with the mycelial mat being cottony, white and dense, and up to 2 cm thick on all media. On MEA, MYPA and MPA the colonies were white and cottony, resembling *Pleurotus ostreatus* mycelium, but not as aerial. Around the inoculum (up to 1-2 cm) more dense mycelium was observed. On PDA and CzA young colonies were also white and cottony, but older colonies on PDA showed zones of brownish mycelia.

3.2 *Growth examination*

The dynamics of mycelial growth of the various strains on five agar media are presented in Figure 2. Three strains (2, 3, 4) grew similarly on all media. The best media for their growth were MEA, MYPA and MPA with an average growth of 3.3 mm/day. The maximum growth rate of the fastest strain, strain 4, was 4 mm/day on MYPA. Strain 1 grew slower than the other strains and optimal media for growth were MPA and CzA at 2.34 mm/day.

The relationship between growth and temperature is shown in Figure 3. No mycelial growth was observed at 6 and 37°C but cultures incubated at these temperatures recommenced growth when incubated at the optimal temperature. It is interesting that we have obtained slow growth of all strains on MEA at 4°C. The study showed that the strains had different temperature optima for mycelial growth. For strain 1 it was between 18 and 23°C with an average growth of

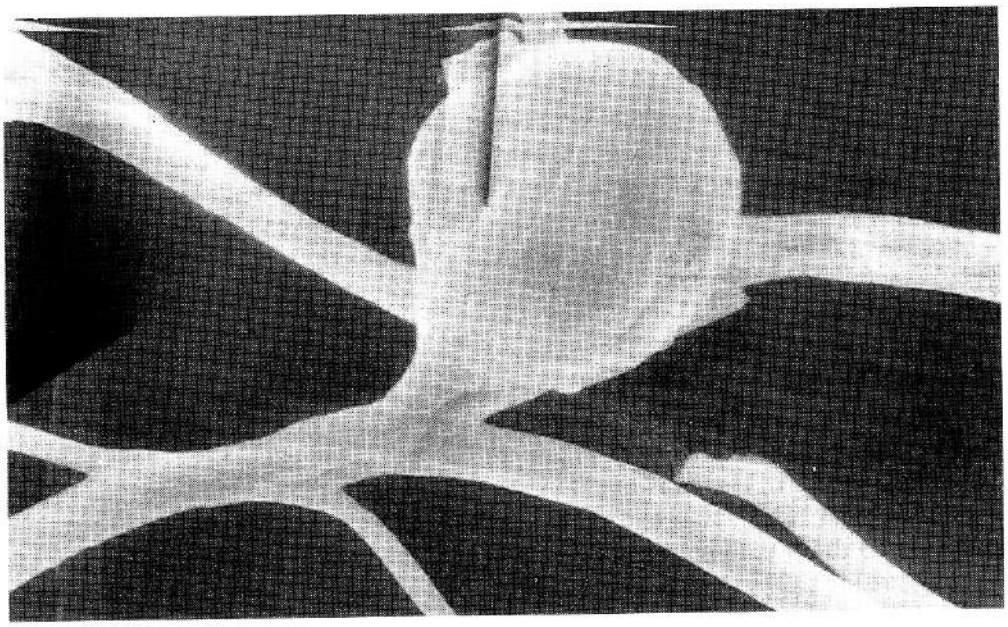

Figure 1. Chlamydospore on hyphae of strain 4 (scanning electron micrograph).

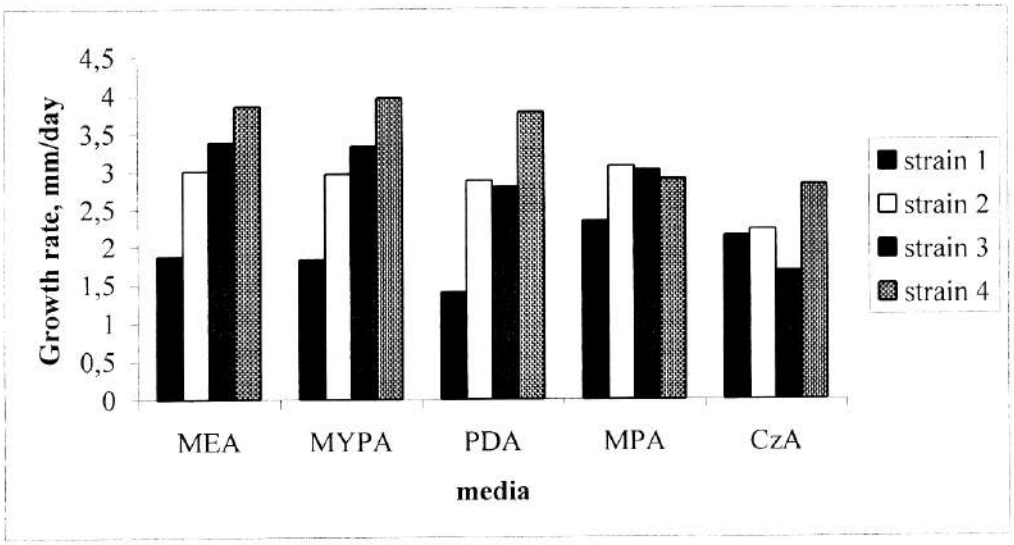

Figure 2. The radial growth rate of *H. marmoreus* strains on different agar media.

2.1 mm/day. The optimal temperatures of growth of the fast growing strains 2, 3 and 4 were between 23 to 27°C, 25°C and 26°C respectively with an average growth rate of 3 mm/day.

The optimal substrate humidity for growth of all investigated strains was between 55 and 66% (Fig. 4). On a substrate with a humidity of 50%, all strains grew slowly. The average growth rate at optimal humidity of rapid growing strains (2, 3, 4) at 26°C was 2.5 mm/day and for the slow growing strain 1 it was 1.9 mm/day.

The concentration of CO_2 raised above the detection limit at day 3. After 9 days of incubation the mycelium covered the most of the substrate and CO_2 concentration rapidly increased (Fig.5).

H. marmoreus strain 4 was the fastest and the best for fruit body production. Strains 2 and 3 commenced harvest four days later. The first flush finished about three weeks after the first primordia started to develop. The distribution and the amount of fruit bodies are described in Fig. 6.

The optimum pH for growth of *H. marmoreus* mycelium (strain 3) was around 6.1 (Table 1). No growth was observed in the acidic region with a pH-level of 3.1. Mycelium grown on the surface of medium 6 at 26°C produced a mycelial mat which covered the surface in 13 days.

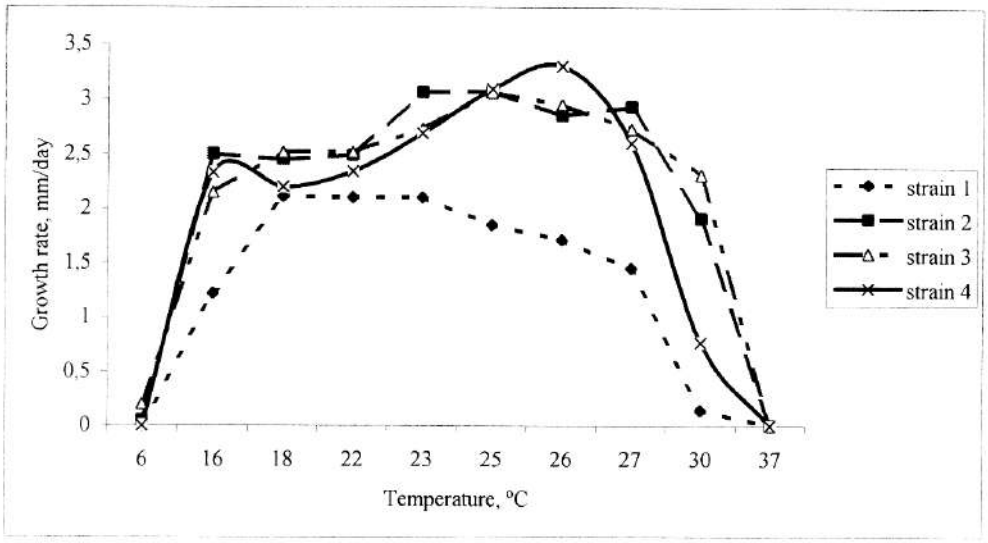

Figure 3. Effect of different temperatures on the growth rate of *H. marmoreus* strains.

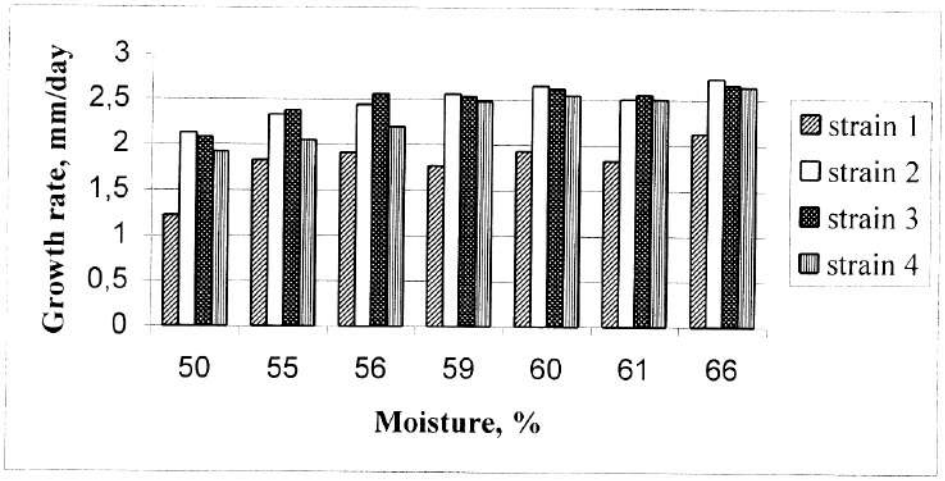

Figure 4. Effects of different moisture contents on the growth rate of *H. marmoreus* strains.

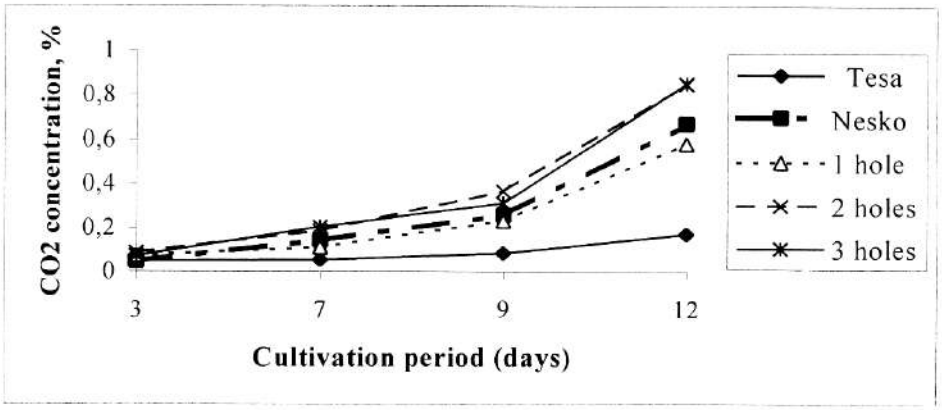

Figure 5. Evolution of the CO_2 concentration in cultures of strain 2.

The maximum dry weight of mycelium grown in medium 6 was 7.5 g/l after incubation for 14 days on a rotary shaker (Fig. 7).

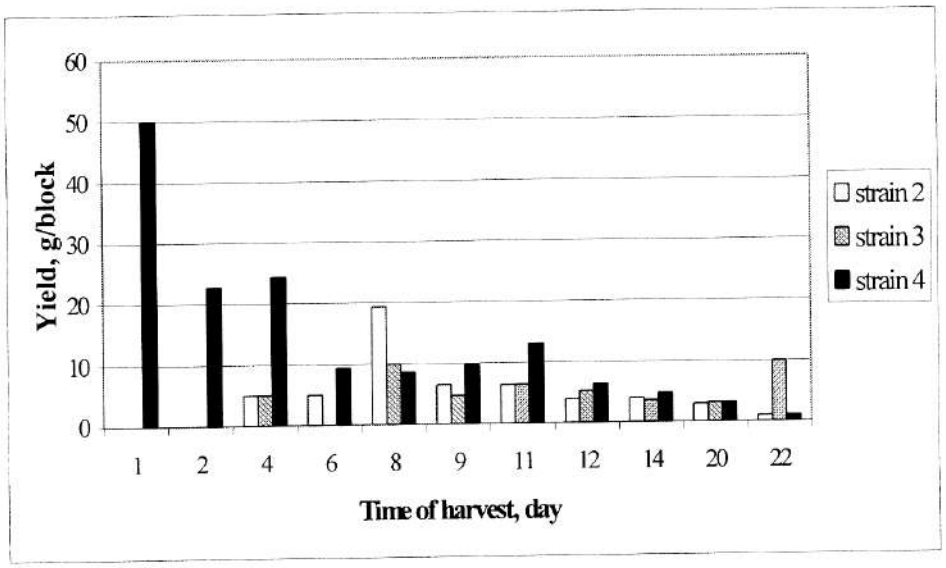

Figure 6. Fruit body production of different *H. marmoreus* strains in the first flush (averages from 20 replicates).

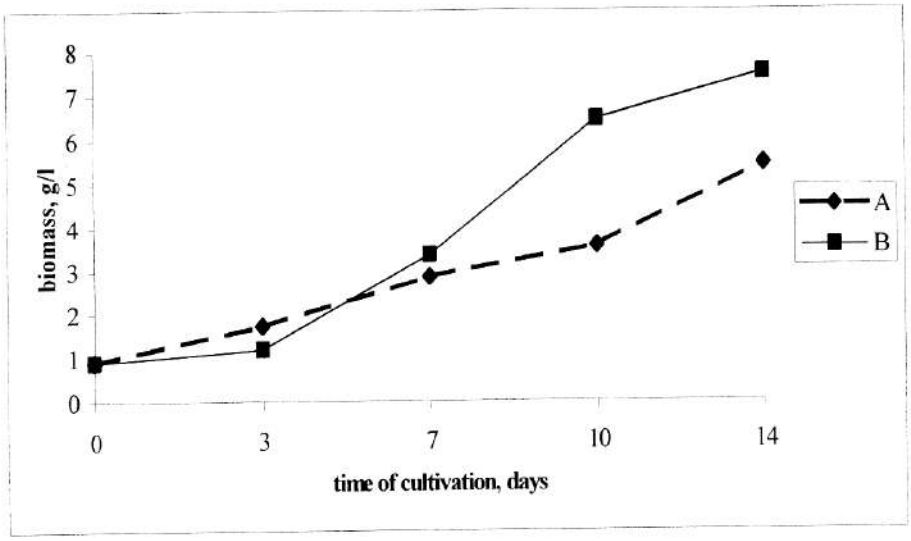

Figure 7. Biomass production of *H. marmoreus* strain 3 in submerged culture.

Table 1. Biomass production of *H. marmoreus* (strain 3) surface cultures on medium 6.

PH-level	pHi*	pHf*	Biomass, g/l
1	6.1	6.5	7.77
2	5.5	5.5	7.62
3	4.9	4.8	6.88
4	4.1	4.3	5.99
5	3.7	4	0.39
6	3.1	3.5	no growth

*pHi –Initial pH of the medium after sterilisation.
pHf – Final pH of the medium after 13 days of cultivation at 26°C.

The data presented in Figure 7 are the mean weights (dry matter, g/l) of the two samples collected. Mycelial pellets were of a maximum size of (diam 2 mm) in medium 6, and smaller

(0.5-1.5mm) in medium 5. Submerged cultures of *H. marmoreus* were covered by surface mycelium within 4-7 days under stationary conditions.

4 CONCLUSION

In culture, vegetative mycelia of *H. marmoreus* produce structures, which are typical of higher basidiomycetes, such as clamp connections, chlamydospores, arthroconidia and crystals. The presence of chlamydospores and arthroconidia is characteristic of dikaryotic mycelium of this mushroom and this has not been described before. On all agar media investigated, mycelial growth was not fast when compared with *P. ostreatus* and *Lentinula edodes*. No large difference in growth rate on agar media and on a solid substrate was observed. This indicates that beechwood sawdust and corn meal substrate provide favourable growth conditions for *H. marmoreus* strains. It is now possible to produce fruit bodies of *H. marmoreus* with high yield in Europe. A few years ago a successful attempt was done in Belgium with the production of fruit bodies of *H. marmoreus* on Shiitake substrate (Peters 1999).

Strain 4 (Japan) was found to be the best of the investigated strains on the basis of mycelial growth rate on agar media, solid substrate, and fruit body production. Strain 1 (European) did not show rapid growth on the studied substrates and was not tested for fruit body production. The influence of temperature and humidity on the mycelial growth for all studied strains of *H. marmoreus* is also typical for the majority of basidiomycetes. We found temperature optima for mycelial growth between 23- 27°C for Japanese strains and between 18-23°C for the European strain. The temperature optimum for mycelium growth of 21 to 24°C obtained by Stamets (1993) does not correspond to our results. The optimal air temperature inside growing rooms for *H. marmoreus* cultivation must be 4 to 6°C lower than inside the blocs of substrate.

The response to the water content of the substrate was very similar for all investigated strains. The optimal range of substrate humidity for vegetative growth of 55 to 66% is also the optimal condition for the production of high quality fruit bodies. The same optimum substrate humidity was found by Grigansky (1999) for *H. erinaceus*.

The CO_2 contents inside the petri dishes depended on the CO_2 production of the mycelium, which degraded the substrate. The production of CO_2 correlated with the growth rate of the investigated strains. After 9 days the CO_2 concentration increased fast and growth of mycelium up to 0.9% CO_2 is possible. Stamets (1993) found that a level above 0.5% CO_2 is required for spawn, but for fruit body development the level must be in the range of 2 to 4%. In our experiments on fruit body production, a CO_2 concentration of 1-2% gave good growth.

The complex medium 6 was better suited for biomass production than liquid malt extract. After 14 days the maximum dry weight of mycelium on media 6 and 5 was 7.5 g/l and 5.5g/l respectively. A pH-level of about 6 was optimal for biomass production. After 14 days of incubation pH had changed a little only, maybe due to the formulation of the nutrient solution.

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