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# Effects of cultivation techniques and media on yields and morphology of the basidiomycete *Armillaria mellea*

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**Summary.** The yield of cell mass and the morphology of *Armillaria mellea,* strain ATCC 11114, was studied using a variety of cultivation methods: solid media, standing liquid culture, shake flasks, tower reactors and impeller-stirred reactors. Two different media, malt extract broth and a glucose/asparagine/peptone-medium, and the corresponding agar media, were used. Yields were higher in the malt extract media than in the glucose media. Generally the highest yields were obtained on solid media while agitated cultures gave the lowest yields. Morphological characteristics such as pellet formation, adhesion to surfaces and pigment production were significantly affected by culture conditions.

## Introduction

The cultivation of filamentous fungi often presents certain problems compared with bacterial and yeast cultivation, e.g. lower growth rates, diffusion limitation due to pellet formation or viscous growth and adhesion to surfaces. However, many lower filamentous fungi (molds) are readily cultured with comparatively rapid growth in liquid or solid state fermentations and some are used in such well known applications as production of certain antibiotics, organic acids, enzymes etc. Much less is known about the cultivation of higher fungi (Basidiomycetes and certain Ascomycetes) which generally are more difficult to cultivate and have considerably lower growth rates. The major application today is the solid state cultivation of edible mushrooms on grain, straw, saw-dust or compost (Chang and Hayes 1978). Mycelia of some edible mushrooms may also be cultivated in liquid media in shake flasks or impeller-stirred fermentors (Worgan 1968; Martin 1983). The most difficult group of fungi to cultivate are the mycorrhizae-formers. Many of these are not yet possible to cultivate in pure culture while others may be grown in liquid media on vermiculite, although the growth rates often are extremely low (Marx and Kenney 1982).

The choice of cultivation vessel and conditions may considerably influence growth rates, morphology and production of metabolites in cultivations of filamentous fungi. However, only few studies have been reported (Wase et al. 1985; Metz 1976) where effects of cultivation techniques are actually compared.

The fungus used in this study, *Armillaria mellea* (honey mushroom), is a common plant pathogenic Basidiomycete which causes white rot in both hardwoods and softwoods. The fungus is variable and, although the name *Armillaria mellea* is well established in the literature, the interpretation of the species varies among taxonomists (Watling et al. 1982). A number of biological species have been included under the name *A. mellea* (Anderson and Ullrich 1979), and this makes comparison of physiological studies using different strains difficult, particularly when isolates originate from different parts of the world.

*A. mellea* forms dark brown zone lines (pseudosclerotial plates) which consist of swollen, pigmented hyphae. Zone line formation in wood is being studied in our laboratory with the aim of developing a process for production of wood whith attractive zone line patterns for use in furniture, floors etc. (Hansson and Seifert is 1987). An important part of this work is to develop techniques for production of active mycelia for use as inoculum on wood.

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In the literature, most reports on the cultivation of *A. mellea* is concerned with its role as a wood pathogen, especially the formation of rhizomorphs (invasive organs). Cultivations have been carried out using solid agar media (Moody and Weinhold 1972; Garraway 1975; Rishbeth 1986) and liquid culture in standing flasks (Garraway and Weinhold 1968; Sortkjaer and Allerman 1972). Measurement of growth rates and yields has been carried out in shake flask culture (Jennison et al. 1955; Hattula and Gyllenberg 1969) and in stirred batch reactors (Eddy 1958). Growth rates and yields seem to be quite variable depending on strains, media, addition of growth stimulators as well as cultivation conditions.

In this study the growth rates and yields of *A. mellea* are compared using a variety of cultivation techniques: standing liquid culture, shake flask culture, tower reactor, impeller-stirred reactor and solid agar media. Two different media are compared, malt extract broth and a glucose medium.

## Materials and methods

*Organism. Armillaria mellea* (Vahl ex Fries) Kummer, strain ATCC 11114 was used in this study. The culture was maintained on malt extract agar slants and kept refrigerated at +4° C.

*Media.* 2% malt extract broth, MEB, (Difco) or a synthetic medium were used. The carbohydrate content of Difco MEB is 91.9% (Zabriskie et al. 1980). The defined medium described by Weinhold and Garraway (1966), with glucose as the carbon source and asparagine as the nitrogen source, failed to support growth of ATCC 11114. Our modification of the medium to include peptone (Difco) allowed the strain to grow, and the medium was therefore called Modified Weinhold (MW), with the following formulation (per litre of deionized water): glucose 15 g, $KH_2PO_4$ 1.75 g, $MgSO_4 \cdot 7H_2O$ 0.75 g, L-asparagine 6 g, peptone 2 g, thiamine 3 mg, and mineral solution 10 ml. The mineral solution contained (per litre of deionized water): $ZnSO_4 \cdot 7H_2O$ 879 mg, $CuSO_4 \cdot 5H_2O$ 39.3 mg, $H_3BO_3$ 5.7 mg, $MnSO_4 \cdot H_2O$ 6.1 mg, and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 36.8 mg. The glucose solution was autoclaved separately and the thiamine solution was sterile filtered through a 0.2 µm filter. The initial pH in MW was adjusted to the same pH, 5.0, as MEB. In agitated cultivations, foaming was controlled by addition of antifoam, Adekanol LG-109 (Asahi Electro Chemical Co. Japan). Solid media (MEB and MWA) were prepared by adding 2% Bacto agar (Difco). Water agar was prepared of water and 2% agar.

*Preparation of stock cultures for cultivation experiments.* A tower reactor (described below) containing 1.2 l of 2% MEB was inoculated with homogenized mycelium from an agar slant. After ten days the pellets formed were centrifuged at 5000 rpm, 5° C for 15 min and washed twice with sterile 0.9% NaCl solution. The pellets were suspended in NaCl solution and then transferred into sterile glass vials with tight lids. The surface of the pellet suspension was covered with approximately 1 cm of sterile mineral oil. The stock cultures were kept refrigerated at +2° C.

*Inoculum preparation.* Homogenized pellets from one stock culture vial were used to inoculate 1.2 l of 2% MEB in a tower reactor. The mycelium was harvested after ten days as described above. Gently homogenized pellets were then used to inoculate each vessel to a final inoculum density of 14 mg dry weight per litre medium. A stomacher (Lab-blender 400, Seward Laboratory, London) was used to homogenize the pellets.

*Cultivation experiments.* All cultivation experiments were carried out at room temperature, approximately 22° C.

*Standing cultures.* Nineteen cm wide Petri dishes were filled with 50 or 400 ml liquid or solid medium to give a medium depth of 2 and 16 mm, respectively. To increase the medium depth to 37 mm, 1 l Erlenmeyer flasks containing 400 ml liquid medium were used.

*Agitated cultures.* Baffled 1 l Erlenmeyer flasks containing 200 ml medium were incubated on a rotary shaker at 115 rpm. The tower reactors were constructed of a Pyrex glass cylinder with an inner diameter of 70 mm and a height of 550 mm. The bottom of the reactor consisted of a porous glass filter through which sterile filtered air was let in. The size of the pores was 20—40 µm. The aeration rate was 0.5 vvm and the medium volume 1.2 l. The impeller-stirred reactor used was a 1.5 l fermenter (Chemoferm AB, Hägersten, Sweden). The operating volume was 1.2 l, aeration rate 0.5 vvm, and stirrer speed 700 rpm.

*Replication.* The heterogeneous suspensions of mycelia obtained in the experiments made it impossible to take representative samples during cultivation. The whole volume of medium in each reactor was used to determine the dry weight, and for this reason the data from 1, 2, 3 and 4 weeks are from different cultivation runs. The cultivations in Petri dishes and Erlenmeyer flasks were performed in duplicate and the cultivations in tower reactors and impeller-stirred reactors were single experiments. All cultivations except those in impeller-stirred reactors were performed twice.

*Determination of dry weight.* Mycelia grown in liquid media were filtered through a sieve with 1.5 mm pores and washed with 0.9% NaCl solution. The loss of mycelium in the filtering procedure was negligible. The mycelia were dried to constant weight at 105° C in glass Petri dishes. Solid media were heated until the agar was completely melted and then filtered through a hot sieve, washed with boiling 0.9% NaCl solution and dried as described above.

*Calculation of yields.* Yield, ($Y_{X/S}$), is defined throughout this paper as g dry weight of cell mass per initial g carbohydrate in the medium.

## Results

Our strain of *A. mellea* grew in Malt Extract Broth (MEB)-media but not in defined glucose-media. Addition of growth stimulators (indole acetic acid, ethanol, acetate) as described by Garraway (1970) and others as well as addition of extracts

(peptone) was tried in initial tests. Of these only peptone addition was successful and was then used in the modified Weinhold medium (MW). *A. mellea* was cultivated in MEB, MW and the corresponding agar media (MEA, MWA). To study the effect of aeration rate as well as shear forces and turbulence the following cultivation techniques were used (in order of increasing aeration rate/shear forces): surface culture on agar (2 different agar dephts) < standing liquid culture (3 different liquid dephts) < shake flask < tower reactor < impeller-stirred reactor.

The yields after 1, 2, 3 and 4 weeks of cultivation are shown in Fig. 1 (solid media), Fig. 2 (standing liquid cultures) and Fig. 3 (agitated cultures).

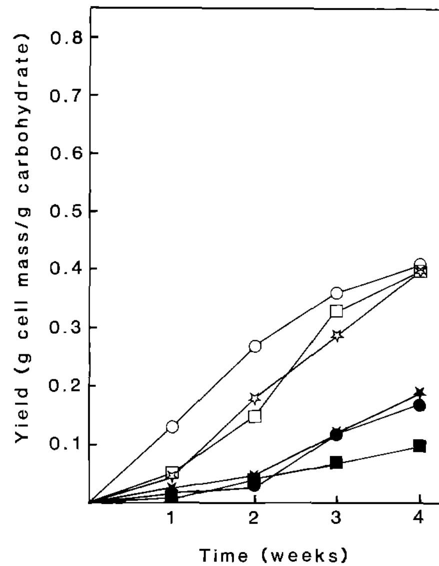


**Fig. 2.** Yields of *A. mellea* in standing cultures in liquid media. Petri dishes with ○ 50 ml MEB; ☆ 400 ml MEB; ● 50 ml MW; ⋆ 400 ml MW. Erlenmeyer flasks with □ 400 ml MEB; ■ 400 ml MW

### *Cultivation on solid media*

The highest yields were obtained in cultivations on solid media, over 0.7 g biomass/g carbohydrate on MEA and around 0.4 g/g on MWA. The decreasing growth after two weeks in dishes with 50 ml MEA was a result of drying up of the medium. *A. mellea* grew mainly on the agar surface with white cottony colonies gradually turning brown, finally forming a brown crust with dark brown liquid droplets. Also rhizomorphs were formed, growing into the agar. Rhizomorphs consist of hyphae which are closely packed together and are thought to play an important role when the fungus invades wood in nature (Sortkjaer and Allerman 1972).

In order to check if this strain of *A. mellea* was able to utilize agar as a nutrient source test tube cultivations were carried out on water agar. No growth was observed after 4 weeks.

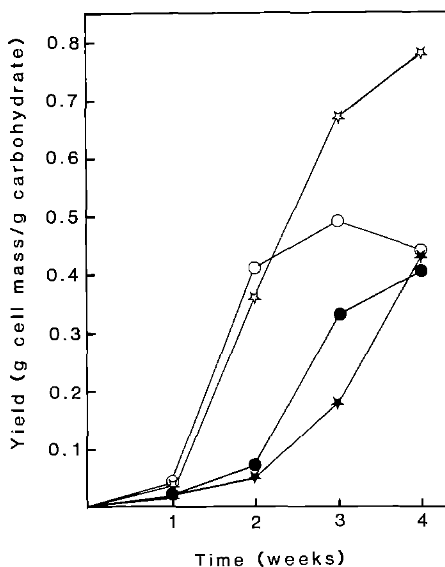


**Fig. 1.** Yields of *A. mellea* in standing cultures on solid media. Petri dishes with ○ 50 ml MEA; ☆ 400 ml MEA; ● 50 ml MWA; ⋆ 400 ml MWA

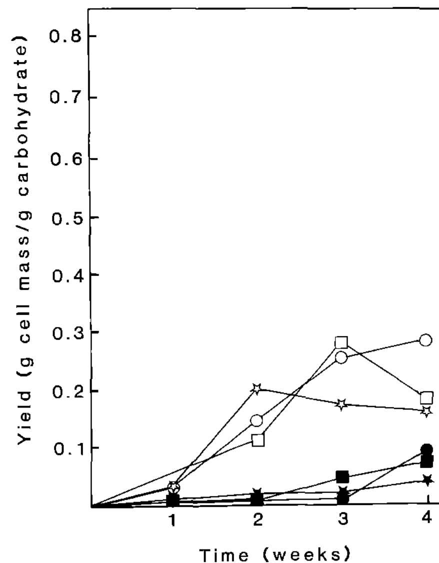


**Fig. 3.** Yields of *A. mellea* in agitated cultures. Tower reactors with ○ 1200 ml MEB; ● 1200 ml MW. Shake flasks with ☆ 200 ml MEB; ⋆ 200 ml MW. Impeller-stirred reactors with □ 1200 ml MEB; ■ 1200 ml MW

*Standing liquid cultures*

Cultivations in standing liquid media were carried out in MEB and MW at liquid depths of 2, 16 and 37 mm, respectively. As on solid media, *A. mellea* grew considerably slower in MW than in MEB. The biomass yields were also lower, around 0.2 g/g in MW compared with 0.4 g/g in MEB. Generally, a shallow liquid depth favoured growth but the differences in growth depending on liquid depth were small compared with the differences due to medium composition.

The mycelium grew partly on the liquid surface, partly submerged in clumps. As on solid media, the surface mycelium was cottony and white, gradually turning brown and forming brown crusts. Submerged, large clumps of rhizomorphs were formed, especially in 16 and 37 mm liquid depths. In some experiments the rhizomorphs were separated from the rest of the cell mass by thoroughly rinsing the rhizomorph clumps. The dry weight of both fractions were then determined. After three weeks of growth in MEB in 37 mm liquid depth around 55% of the total cell mass consisted of rhizomorphs.

*Agitated cultures*

Cultivations were carried out in MEB and MW in shake flasks, tower reactors and impeller-stirred reactors. The difference in yields between the two media was even more pronounced in agitated cultures than in standing liquid and solid media cultures. The highest yield, around 0.3 g/g, was obtained in MEB in tower reactors while growth was insignificant in MW during the first 3 weeks in any of the agitated cultures.

The culture morphology was affected by culture conditions. In shake flasks, part of the mycelium grew on the glass walls and the rest formed hard pellets in the medium. In tower reactors a dense suspension (in MEB) of soft pellets, around 2—5 mm diameter, were formed. No wall growth occurred but the glass filter tended to become covered with mycelium resulting in larger air bubbles. In the impeller-stirred reactors the mycelium grew mostly on the glass walls, on the impeller and behind the baffles with only few pellets in the medium. The texture of the mycelium was very hard and dense. In the flask cultures and tower reactors the medium gradually turned light brown. In the impeller-stirred reactor the pigmentation was more intense giving an opaque, black-brown liquid after 2 weeks. In all agitated cultures the mycelia grew only as pellets or attached to surfaces and no rhizomorphs were formed.

## Discussion

A comparison between the yields of cell mass of *A. mellea* obtained in our experiments and some growth data reported in the literature is shown in Table 1. The defined glucose-medium described by Weinhold and Garraway (1966) has frequently been used for cultivation of different strains of *A. mellea*. Either asparagine or $(NH_4)_2HPO_4$ has been used as nitrogen source. The ATCC strain used in our investigation did not grow in the defined medium. In the modified Weinhold medium (MW, MWA) which included glucose, asparagine and peptone moderate growth of *A. mellea* was obtained but malt extract broth/agar was a clearly better medium. Our yields obtained on MWA are comparable to the values for solid media reported by Garraway (1975) and Moody and Weinhold (1972). In the latter paper a yield of 0.77 g cells/g glucose was reported but in this case large amounts (50 g/l) of lecitin had been added and part of this compound may have been used as a carbon source.

In standing liquid culture literature data suggest low yields in glucose media without growth stimulators but substantially higher yields when anthranilic acid, ethanol, indole acetic acid or acetate was added. Especially addition of ethanol resulted in high yields, 0.6 g/g (Garraway and Weinhold 1968). In our experiments using the ATCC strain neither ethanol, indole acetic acid nor acetate stimulated growth. Peptone did stimulate growth but not as efficiently as ethanol and other stimulators in the literature reports. High yields with our ATCC strain was obtained only in MEB-media.

Variations in growth characteristics between strains are to be expected. The strains of *A. mellea* used in experiments reported in the literature were isolated in different parts of the world, from different substrates and may not even represent the same biological species. In our own experience (unpublished data), when studying own isolates of *A. mellea* from our region, considerable variation is usually noted in growth rates, colonial appearance and pigmentation. In our cultivations of the ATCC strain of *A. mellea* the cultivation techniques clearly affected growth rates and yields. The highest yields were obtained on solid media, lower yields in standing liquid culture and

**Table 1.** Comparison of yields in cultivation of *A. mellea*

| Cultivation method | Carbon/nitrogen source (g/l) | Additional substance | Cultivation time (days) | Yields (g/g) | Reference |
|---|---|---|---|---|---|
| Solid medium | glucose 20/asparagine 4 | — | 21 | 0.10 | Garraway 1975 |
| Solid medium | glucose 20/asparagine 4 | anthranilic acid 20 mg/l | 21 | 0.25 | Garraway 1975 |
| Solid medium | glucose 10/asparagine 2 | — | 35 | 0.18 | Moody et al. 1972 |
| Solid medium | glucose 10/asparagine 2 | lecitin 50 g/l | 35 | 0.77 | Moody et al. 1972 |
| Standing liquid | glucose 5/$(NH_4)_2HPO_4$ 2 | — | 21 | 0.05 | Garraway et al. 1968 |
| Standing liquid | glucose 5/$(NH_4)_2HPO_4$ 2 | EtOH 500 ppm | 21 | 0.60 | Garraway et al. 1968 |
| Standing liquid | glucose 5/$(NH_4)_2HPO_4$ 2 | — | 21 | 0.06 | Sortkjaer et al. 1972 |
| Standing liquid | glucose 5/$(NH_4)_2HPO_4$ 2 | acetate 10 mM | 21 | 0.31 | Sortkjaer et al. 1972 |
| Agitated | MEB | — | 20 | 0.03 | Jennison et al. 1955 |
| Solid medium | glucose 15/asparagine 6 | peptone 2 g/l | 21 | 0.18—0.33 | This report |
| Solid medium | MEB 20 | — | 21 | 0.49—0.68 | This report |
| Standing liquid | glucose 15/asparagine 6 | peptone 2 g/l | 21 | 0.07—0.12 | This report |
| Standing liquid | MEB 20 | — | 21 | 0.28—0.36 | This report |
| Agitated | glucose 15/asparagine 6 | peptone 2 g/l | 21 | 0.02—0.04 | This report |
| Agitated | MEB 20 | — | 21 | 0.17—0.29 | This report |

the lowest yields in agitated culture. These relations were found in both media, although the yield levels were different.

Various factors may influence growth conditions (when medium composition and inoculation density are the same), e.g. limitation of oxygen or nutrients, diffusion rates, growth rates of submerged versus aerial mycelia and effects of shear stress. The aeration rate is not likely to be of critical importance in these experiments. One reason is the low growth rate of higher fungi compared with e.g. molds which grow rapidly in the same kind of cultivation equipment. Another reason is that higher fungi, especially wood decomposers, are known to grow well at low oxygen tensions, even microaerophilic conditions (Gundersen 1961). In our experiments (Hansson and Seifert 1987) *A. mellea* was found to grow (on MEA) almost as well in 1% oxygen as in air.

Whether, as in this case with *A. mellea*, growth rates are higher when fungi grow mostly as aerial mycelium on surfaces compared with submerged growth is an interesting and more general question. Fungi grow apically in the hyphal tips, and it is also well known that during submerged growth in clumps or pellets a large part of the mycelium is inactive (Wittler et al. 1984). One may therefore speculate that surface growth may be faster since a larger part of the mycelium is active resulting in a greater number of growing tips. A source of experimental error is possible when using solid media if yield calculations are based on added carbon source and the fungus at the same time may utilize the agar as carbon source. Some fungi may grow on water agar but this was not the case with our strain of *A. mellea.* However, part of the nitrogen source, asparagine, may have been utilized as a carbon source. Shear stress may be an explanation why the lowest yields were obtained in agitated cultures. Only the cultivation on MEB in tower reactors gave comparatively high yields. The effect of shear stress was particularly evident in impeller-stirred reactors where the fungus seem to "avoid" turbulence by forming very hard mycelial clumps attached to surfaces but almost no dispersed growth. The intense pigment production may be interpreted as a defense mechanism. Many fungi, among then *A. mellea*, are known to produce highly pigmented hyphae in pseudosclerotial plates as a defense mechanism against unfavourable environmental conditions (Nelson 1973). Also Jennison et al. (1955) reported poor growth of *A. mellea* in agitated culture. Other higher fungi, such as *Polyporus* sp. and *Pleurotus* sp. appear to grow well during highly turbulent conditions (Carroad and Wilke 1977) although biological changes such as dedikaryotization may take place (Ginterova 1973). Some lower fungi may respond to shear stress by growing in the yeast-like form rather than the filamentous form (Solomons 1980).

In conclusion, the production of mycelial biomass of *A. mellea* is highly dependent on the media and strain used. The highest yields were obtained in surface cultivation but if larger amounts of mycelia are needed, submerged cultivation in tower reactors should be the most convenient cultivation method.

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