

Culture Maturity of *Lentinula edodes* on Sawdust-Based Substrate in Relation to Fruiting Potential^{*1}

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표고 톱밥배지의 성숙도와 자실체형성 포텐셜^{*1}

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ABSTRACT

Culture maturity assessment can be used to control fruiting body flush timing. Culture maturity of sawdust-based substrate was evaluated by using oak mushroom, [*Lentinula edodes* (Berk.) Pegler]. The influence of substrate water potential (ψ) on the growth and fruiting of three genotypes of *L. edodes* was also investigated.

Glucosamine content revealed a peak at the fruiting body senescent stage. Glucosamine increased steadily to the sporophore senescent stage, and sharply declined at crop treatment. Lipid phosphate and ergosterol contents peaked at pinning and button break stages, respectively. Therefore lipid phosphate and ergosterol contents would be considered as the convenient measurement for judging culture maturity and fruiting potentials. The substrate pH values before inoculation and on the fruiting stage were varied from 6.3 to 4.0. This pH changes were detected as changes in color from bluish purple to yellow by direct bromphenol blue(BPB) spraying, and shown a good correlation with fruit body yield of the 1st flush.

Concerning water potential of the cultures, a slight reduction of water potential, -0.5MPa, stimulated mycelial and colony growths on liquid, agar and sawdust-based substrates. The water potential of well-colonized matured substrate was -0.7MPa and -4.0MPa, before and after the fruiting, respectively. Excellent water providing capacity (higher ψ) is expected to well-matured cultures with a high density of mycelial colonization. Also, the substrate water potential significantly affected by the interaction between genotypes and spawn run time.

Keywords: Culture maturity, *Lentinula edodes*, Sawdust-based Substrate, Fruiting, Glucosamine, Ergosterol, Water potential, Water holding capacity

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- 요약 -

본 연구는 표고의 톱밥재배시 재배배지의 속도, 자실체 발생단계 및 버섯생산 수량을 정량화하고 예측하고자 실시하였다. 배지의 성숙도는 자실체 발생시기를 결정하는데 매우 유용한 지표로서, 균주, 영양생장기간, 여러 가지 효소의 활성이 관여하며, 배지내의 성분변화, pH 변화 및 수분포텐셜 등이 영향을 미친다. 자실체 발생 초기에 배지내의 glucosamine의 함량이 최고치를 나타냈으며, 자실체 발생후 신속히 감소하였다. lipid phosphate 및 ergosterol 함량도 버섯원기 형성시에 높은 값을 보였으며, 자실체 발생직전 최고값에 달하였다. 따라서 lipid phosphate 및 ergosterol 함량이 배지의 속도 및 자실체형성을 예측하는 주요수단으로 사용될 수 있다. 기질내의 pH는 종균접종전 6.3으로부터 자실체형성단계에서 4.0으로 변화하였으며, pH측정으로 자실체수량을 예측할 수 있었는데, 이러한 pH를 bromphenol blue(BPB)의 스펙트로 간단히 측정할 수 있었다. 아울러 기질의 수분포텐셜과 관련하여, -0.5MPa 정도의 수분포텐셜감소가 생장 및 자실체형성에 크게 영향하였으며, 우수한 속도의 버섯재배 기질이 가지는 수분포텐셜은 버섯발생 전후 각각 -0.7MPa, -4.0MPa였다. 높은 수분포텐셜은 고밀도의 균체콜로니를 만들 수 있었으며, 균주 및 배양기간중의 상호작용이 기질의 수분포텐셜에 현저히 영향하였다.

INTRODUCTION

Oak mushroom [*Lentinula edodes* (Berk.) Pegler] cultivation on sawdust-based substrates has two distinct phases: a vegetative growth phase and a fruit body formation phase. The physiological activity of oak mushroom differs between the phases. Thus, the crop is differently managed for each phase. During the vegetative phase, sterilized and inoculated substrates are contained in a vented polypropylene bag, and the mycelium of oak mushroom colonizes the substrate within the bag with knitting it into a solid block. The culture matures as the mycelia break down the substrate, and stores nutrients for subsequent fruit body formation. The fully colonized matured blocks removed polypropylene bag are placed under fruiting conditions to promote mushroom production, which is called as a fruit body formation phase.

Growth and maturity of oak mushroom cultures are influenced by many factors. These include substrate moisture content (Matsumoto and Kitamoto, 1987), water potential of the substrate (Ohga *et al.*, 1998; Ohga, 1999), genotype (Diehle and Royse, 1986; Ohga, 1998), substrate constitutions (Royse and Bahler, 1986; Ohga, 1990), and an atmosphere during the vegetative phase (Leatham and Stahmann, 1987;

Donoghue and Denison, 1995). The length of the vegetative stage (Royse, 1985; Ohga *et al.*, 1992) and the activities of various enzymes (Ohga, 1992) also have been shown to directly affect the fruiting capacity of oak mushroom.

The water potential (ψ) of a cell is the sum of the osmotic potential (ψ_{π}) and turgor pressure. The difference in water potential between substrate and mycelial cell strongly influences water uptake by the mycelium. The water potential of the substrate depends on the concentration of solutes in the substrate water. This concentration is influenced by substrate additives and solutes formed from the fungus mycelium. The metabolism of mycelium in such substrates produce metabolically water through its hydrolysis. This metabolism results in a rise water potential around the primordium. These hydrolysis products act as solutes to decrease water potential in a substrate. The higher concentration of such solutes in the external matrix, the more difficulties for fungal cells to build up a water supply to the external solutions. Breakdown products of the substrate produced by mycelial extracellular enzymes and excreted metabolites can increase the concentration of solutes in the substrate, which lower its osmotic potential and make water uptake more difficult for the cells. A water potential gradient in the hyphae with

the lower potential in the fruit bodies could be the basis for a translocation mechanism to pressure-driven mass flow. In the fruit bodies, a low potential could be generated osmotically and by transpiration (Kalberer, 1987).

Water uptake by a mycelium depends on the water potential difference between the mycelium and the external solution, on the mass of the mycelium and the matric potentials in substrate. Only few percentage of the total water of the substrate is available to *L. edodes* mycelium for growth, while the rest is bound up to the chemical structure of the substrate and is unavailable. Matrix binding water to the substrate particles is more strong, in the case of the low moisture content of sawdust-based substrate. In the sawdust-based substrate this factor exerts the major effect on water availability to the growth and fruiting of *L. edodes*. A little decreases in water potential at final vegetative growth phase had positive effects on flush quantity. A high water potential during fruiting process resulted in a good primodium development. Therefore, chemical constitution, moisture content, water potential of the substrate, genotype, length of the vegetative stage, activities of various enzymes, timing of bag removal, and its induction of fruit body formation strongly influence mushroom production. To optimize mushroom yield, incubating cultures must be at the proper maturity stage when induced to shift from the vegetative phase to the fruiting phase. Assessment by appearance, based on the growers' experiences, is the only method currently available to determine culture maturity.

This study was performed to evaluate the culture maturity of sawdust-based substrates with various methods of quantifying oak mushroom, [*Lentinula edodes* (Berk.) Pegler], and to characterize for the potential mushroom yield. The influence of substrate water potential (ψ) on the growth and fruiting of three genotypes of *L. edodes* was also investigated.

MATERIALS AND METHODS

Strains

Twelve strains of *Lentinula edodes* belonging to three fruiting types were used (these were maintained in the Kyushu University Forests culture collection). Strains KS-5, KS-9, KS-10, and KS-46 are wide-range temperature types. Strains KS-43, KS-53, KS-58, and KS-60 are warm-temperaure types. Strains KS-6, KS-22, KS-24, and KS-50 are low-temperature types. All of them are used extensively in Japanese commercial cultivation of *L. edodes*.

Culture media and growth conditions

A sawdust-based medium containing 70% (dry wt) *Quercus mongolica* sawdust (20-40mesh), 10% wheat bran, 10% rice bran, and 10% corncob meal was prepared with addition of water to give a final moisture content of 60% (wet wt basis). Polypropylene bags were filled with the sawdust-based medium to 1.2kg (wet wt), and autoclaved at 120°C for 30min. After cooling to room temperature, the medium was inoculated with 10g of sawdust spawn. The bags were then sealed with micro-porous filter caps and placed in a controlled environment for incubation at 20°C for 80 days. After mycelia ran through the substrate, the bags were exposed to dim light of 500 lux.

After 80 days of incubation, cultivation bags were completely removed, and the blocks moved to a flushing room maintained at 17°C. Mist was provided for 1hr daily by overhead nozzles. Relative humidity was maintained at 85% during this period. Sufficient air change was provided to keep the CO₂ level below 1500ppm. Light intensity of 1000 to 2000 lux was provided for 12hr per day by cool white fluorescent bulbs.

Sampling procedure

Samples of sawdust-basted cultures were taken from duplicate cultures, arbitrarily chosen

from fruiting blocks and non-fruiting blocks, respectively. Sampling was done every 2 days throughout the fruiting stages during the first flush period.

Glucosamine content

Chitin was assayed by the method of Braid and Line (1981). For fungal chitin hydrolysis to N-acetyl glucosamine, the dry culture was incubated with HCl. Colorimetric assay was done using MBTH and iron (III) chloride.

Ergosterol content

The ergosterol extraction procedure was modified from Seitz *et al.*, (1977). The saponification was carried out with methanol, ethanol and KOH. Ergosterol was measured by high-performance liquid chromatography.

Lipid phosphate content

The chloroform-methanol extraction procedure of Bligh and Dyer (1959) was used to assay for lipid phosphate. After digestion in 30% perchloric acid, inorganic phosphate was measured colorimetrically at 830nm, using the molybdate blue reaction (Dittmer and Wells, 1969).

Coloration produced by spraying with pH indicators

The pH indicators were bromphenol blue (BPB, a yellow color at pH 3.0 and bluish purple at 4.6), bromocresol green (BCG, a yellow color at pH 3.8 and blue at 5.4) and combined indicator of methyl orange with indigo carmine (MO+IC, changes from red to yellow at pH 4.1). All reagent concentrations were 1 g/L in 95% ethanol except for IC, which was 2.5 g/L in distilled water. The pH indicators were sprayed directly by a handy sprayer to the cut surfaces of sawdust-based cultures. The immediate color change and that after 30min were the most useful periods. Color change was recognized immediately by naked eye, and was then mea-

sured with a Minolta CR-200 Colorimeter (Ohga, 1985). The color was also determined to Muncell Numbers with a Color Chart. Color phase can be obtained by L*a*b* mode (CIE-1976): a* value: reddish degree, b* value: yellowish degree. The ΔE^*ab values was obtained as follow: $\Delta E^*ab = \{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2\}^{1/2}$. The pH value of cultures were measured on sawdust-based culture mixed with deionized water by the ratio of 1 to 20.

Water potential (ψ) measurement

Thermocouple psychrometry, a Wescor HR-33T microvoltmeter coupled to a C-52F sample chamber, was used to determine water potential of agar and sawdust-based substrate samples. Cooling for 15sec of the thermocouple junction and an equilibrium time of 30sec were used for each sample. All measurements were made in the dew point mode, after calibration with a series of Whatman filter disks dipped with NaCl solutions of known potentials. The sawdust-based samples were sealed for 10 min into a Decagon SC-10 sample chamber for equilibrium. Moisture content of sawdust-based substrate was determined by oven drying method.

RESULTS and DISCUSSION

The mycelial growth of *L. edodes* is a lengthy and complex process involving the use of nutrient additives and lignocellulosic complex such as broad-leaved tree sawdust followed by a long fruiting period. When a fungal biomass cannot be separated from a solid culture, growth of the fungus can be monitored by measuring a chemical component. In earlier experiments by Wood (1979), the weight of mushroom mycelium in fully colonized compost was estimated using an extracellular laccase activity, and calculated to be 50-125mg/g compost. Tests for effective indicators of fungal mass must be sensitive, reliable, and time-efficient. Total biomass and

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fungal biological activity were estimated by biochemical tests, such as contents of glucosamine, ergosterol, phospholipid, and esterase activities (as measured by FDA hydrolysis). They were monitored to evaluate culture maturity and fruiting potential reflected to fungal activity during fruit body development stages.

Glucosamine

Chitin is a poly-N-acetylglucosamine with the monomers connected by α , 1-4 links in a straight chain, and is one of the most frequently occurring polymers in fungal wall. Blumental and Roseman (1957) surveyed 25 fungal strains and found that their chitin content ranged from 2.6% (*Neurospora crassa*) to 26.2% (*Aspergillus parasiticus*) of the dried mycelial weight. Zonneveld (1971) reported that the cell wall of *Aspergillus nidulans* contained 19.1% chitin on a dry-weight basis. Sharma *et al.* (1977) reported that the accuracy of glucosamine assay technique for determination of fungal biomass in any tissue depends upon a reliable constant or conversion factor relating hexosamine content to unit dry weight of mycelium. The proportion of glucosamine to the mycelium weight varies with age, morphology and the environment in which the fungus grows.

There were distinct differences in glucosamine contents between the fruiting and non-fruiting cultures (Fig. 1, (a)). The glucosamine contents were increased gradually from days 0 to 12th, the stage C (colonized) to the stage S (senescent), with a subsequent declined day 14th, stage H (harvest) in crop treatment. These changes were observed in both of two strains, KS-9 and KS-60. Glucosamine contents of non-fruitings did not show the same trends. No differences were observed between fruiting cultures and non-fruitings at the C stage. The rapid increase of glucosamine on the fruiting cultures probably derived from the cell lysis or the release of proteins from the mushroom fungus.

Ergosterol

Ergosterol is the predominant sterol component of most fungi, and is either absent or a minor constituent in most higher plants. In extracts of plant materials containing other sterol, fungi ergosterol can be measured specifically on the basis of its characteristic UV absorbance. Ergosterol has been used qualitatively to indicate fungal colonization in a number of materials, such as seeds, leaves and woods (Seitz *et al.*, 1977; Miller *et al.*, 1983; Forbes *et al.*, 1989). It has also been employed to quantify biomass in several studies of soils and mycorrhizal system (West *et al.*, 1987; Lumsden *et al.*, 1990; Martin *et al.*, 1990; Jhonson and McGill, 1990b). In *Agaricus bisporus*, grown on malt extract broth and *Hebeloma crustuliniforme*, grown on Melins-Norkrans medium, a linear relationship was found between ergosterol content and biomass (Matcham *et al.*, 1985; Jhonson and McGill, 1990a). Nine species of aquatic hyphomycetes were investigated by Bermingham *et al.* (1995), and there were correlated between ergosterol content and biomass for three of them, and no apparently correlated for six of them. Besides varying between species, ergosterol content also varied with age of mycelium, although this did not follow any simple pattern. As incubation time increased, there was an increase in the number of senescent hyphae and substantial amounts of biomass formed in the conidia stage (Suberkropp, 1991).

The changes in ergosterol content were shown in Fig. 1 (b). Two phases were observed in the fruiting cultures. First, the ergosterol contents rapidly increased to reach a maximum at 8th day. This phase just stopped at stage B (button), after beginning of fruiting stage. Second, it was decreased rapidly according to the sporophores ripeness. The ergosterol contents were reached to 2,000 $\mu\text{g/g}$ of fruiting culture, but no significant differences between two strains, KS-9 and KS-60, were observed. Ergosterol content of non-fruiting culture did not vary to any great

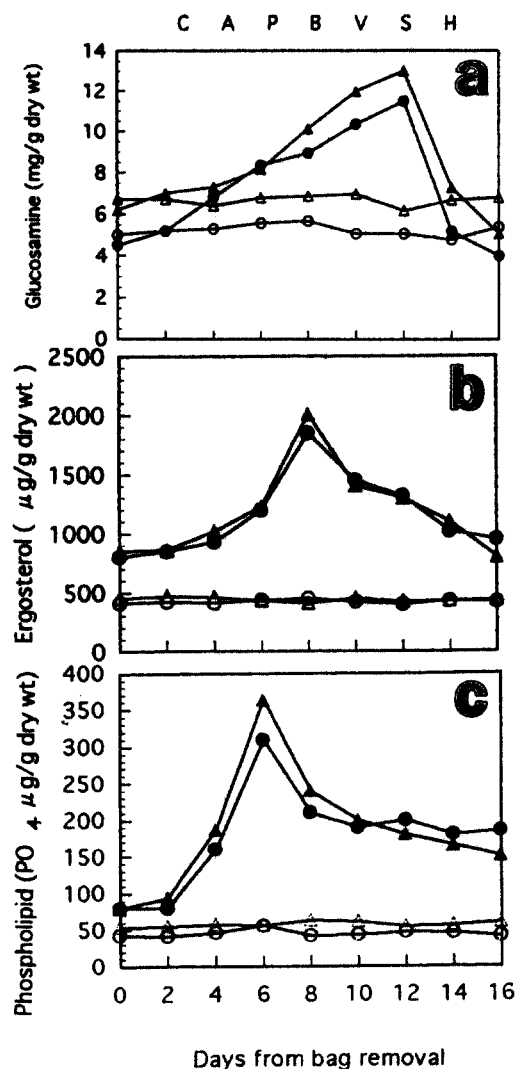


Fig. 1. Changes in various components of sawdust-based cultures during development stages. (a) Glucosamine, (b) Ergosterol, (c) Phospholipid. ● fruiting culture of KS-9; ▲ fruiting culture of KS-60; ○ non-fruiting culture of KS-9; △ non-fruiting culture of KS-60.

extent. A clear distinction was recognized between fruiting and non-fruiting cultures at the stage C. The ergosterol contents of fruiting culture were $800 \mu\text{g/g}$, and its values were

about two-fold higher than non-fruiting cultures at this fully colonized stage. This result indicated that the ergosterol of mycelial stage increased during the period of fruit body initiation, and then immediately declined after the onset of reproductive fruit body growth, following vegetative phase.

Phospholipid

The phospholipid contents reached a peak and started to decline much earlier than the ergosterol. At this stage B when majority of the phospholipid was being utilized, the construction of cell units in fruit body tissue may be reached a peak. The trend of microbial activity during fruiting was quite similar with results expressed to ergosterol or phospholipid contents of cultures. The phospholipid values were quite higher (1.5-fold) in the fruiting cultures than the non-fruiting ones at the stage C. The phospholipid content of the culture increased from an initial value of $80 \mu\text{g}$ to $320 \mu\text{g PO}_4$ (KS-9) or $360 \mu\text{g PO}_4$ (KS-60)/dry weight during the first 6th day (pinning stage, P), and then rapidly decreased during the following 10th day of fruiting stage (Fig. 1, (c)). Pinning stage contained 4 to 4.5-fold higher biomass contents compared with the colonized stage.

Relationship between b^* value by BPB staining and fruit body yield

Fruit body yields were estimated by the test of coloration on spraying BPB indicator. Relationship of the b^* values and fruit body yields on 1st crop was recognized as positive (Fig. 2). High b^* value was correlated with culture maturity, and this high value was also one of the indicators of high quantity of fruit body formation. The colorimetric method was a useful determining method for the culture maturity. Although all strains produced fruit bodies, the biological efficiency (fresh weight of fruit body/dry weight of substrate multiplied by 100, %) varied from 27 to 50% (KS-24), 30 to

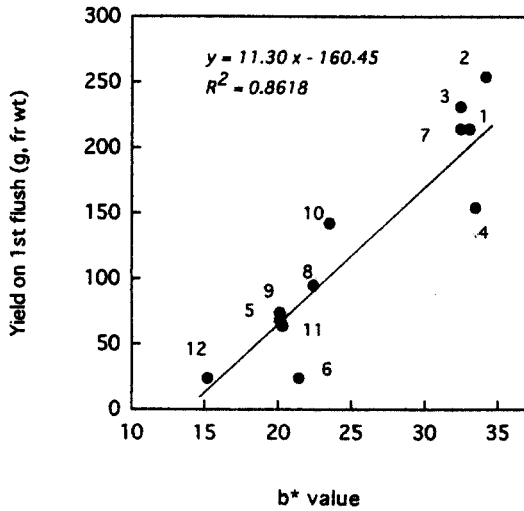


Fig. 2. Relationship between b^* values and fruit body yield on the first flush. The b^* values were measured on day 90th just before fruit body initiation. Strain types according to temperature preference are as follows: wide-range strains (1, KS-5; 2, KS-9; 3, KS-10; 4, KS-46), warm-weather strains (5, KS-43; 6, KS-53; 7, KS-58; 8, KS-60), cold-weather strains (9, KS-6; 10, KS-22; 11, KS-24; 12, KS-50).

58% (KS-58) and 33 to 61% (KS-9) for the spawn-running time (period of vegetative growth stage), respectively (Fig. 3). Significant differences in biological efficiencies were found among the 3 strains evaluated in 1st, 2nd and 3rd flushes over 160 days of production period.

Effect of water potential(ψ) on fruiting

It is generally acknowledged that the water holding capacity of a substrate is related to the culture age. Excellent water providing capacity (higher ψ) is expected in the substrate of well-matured stage with a high density of mycelial colonization. During fruiting, high humidity level needs to reduce the evaporation from the substrate, hence promote the primordia formation. The better growth of basidiocarps can

Fig. 3. Fruit body yields for three-time flushes of *L. edodes* cultivated on sawdust-based substrate. Shaded bars, 1st flush; open bars, 2nd flush; hatched bars, 3rd flush. Spawn-running times for vegetative mycelial growth stage are as follows; A, 70; B, 75; C, 80; D, 85; E, 90 days.

be correlated with higher atmospheric humidity, and mycelial growth is extremely changed with moisture content of substrate. The mycelium of *L. edodes* has quite high water absorbing potential, which results in a high moisture contents in the colonized substrate. Pinning and pinhead development require higher water potential levels in order to encourage free water transport from the culture to the primodium. The lower water potential of the substrate requires more energy consumption for water uptake by the fungus. To obtain good yields of fruit bodies as well as its good quality, water potential of the substrate has to be high and constant during fruit body growth.

The fruit body of *L. edodes* normally contains more than 90% water. The moisture content and water potential of the substrate decrease markedly during the growth of a high yielding flush. Therefore, the water supply strongly influences both the quality and quantity of sporophores produced. The mycelium takes a major fraction

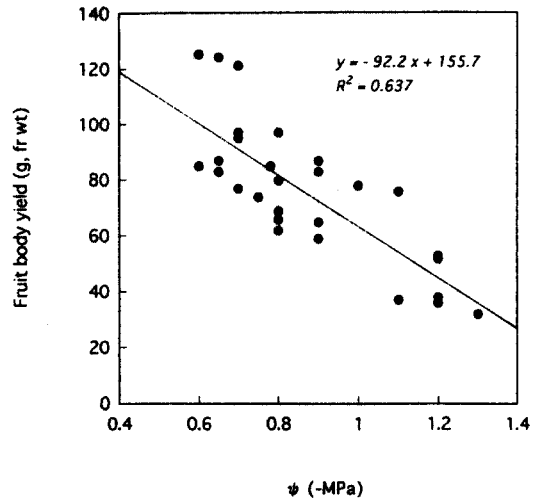


Fig. 4. Effect of soaking treatment on the water potential of sawdust-based substrates. Shaded bars, before soaking of 2nd flush; open bars, after soaking of 2nd flush; cross-hatched bars, before soaking of 3rd flush; vertical/horizontal hatched bars, after soaking of 3rd flush. Data are expressed as mean \pm standard deviation of four replicates.

Fig. 5. Relationship between water potential and fruiting body yields. The water potentials of various substrates were measured after soaking, just before fruiting. The strains were KS-24, KS-58, and KS-9.

of its water for fruit body formation. Soaking of the substrate in water between flushes is essential to optimize production. Misting alone is not sufficient for primordia formation, because of the quite low water potential after picking of fruit bodies. Mushroom cultures lose water by harvesting of the fruit bodies, evaporation and transpiration from the surface of substrate. The water potential was -0.7 to -1.2 MPa after soaking treatment (Fig. 4). The substrate water potential significantly affected by the interaction between genotypes and spawn run time. There was a marked decrease of water potential in the sawdust-based substrate after every flush. The water potential decrease was larger in high yield strain, KS-58, compared to lower yield strain, KS-24. As shown in Fig. 5, positive relationship was found that sawdust-based substrates with higher water potentials resulted in higher fruiting ability.

CONCLUSIONS

Chemical constitutions and water potential of the substrate, genotype, length of the vegetative stage, activities of various enzymes, timing of bag removal and its induction of fruit body formation strongly influence mushroom production. To optimize mushroom yield, incubating cultures must be at the proper maturity stage when induced to shift from the vegetative phase to the fruiting phase. The substrate maturity is closely linked to control the fruit body flush timing. Maturity assessment by substrate appearance based on experiences, is the only method currently available to determine its maturity. This study was performed to evaluate the culture maturity of sawdust-based substrates with various methods of quantifying oak mushroom, [*Lentinula edodes* (Berk.) Pegler], and to characterize for the potential mushroom yield.

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steadily to the sporophore senescent stage, then declined sharply crop treatment. Lipid phosphate and ergosterol contents showed a peak at pinning and button break stages, respectively. The both contents were quite high at the fully colonized stage prior to fruiting, and rapidly increased on the fruiting culture. The pH values of the cultures were varied from 6.3 of before inoculation to 4.0 on the fruiting stage. Culture pH changes were detected as changes in color from bluish purple to yellow by direct spraying Bromphenol blue (BPB). This direct BPB staining were shown a good correlation with culture maturity as well as fruit body yield of the 1st flush.

Concerned to the substrate water potential(ψ) on the growth and fruiting of three genotypes of oak mushroom, the water potentials were significantly changed by the interaction between genotypes and spawn-running time. It has been found that this mushroom grew well at about -0.5MPa water potential which corresponds to moisture contents of 55%. A slight reduction of water potential, -0.5MPa, stimulated mycelial and colony growths on liquid, agar and sawdust-based substrates. The water potentials of well-colonized matured substrates were -0.7MPa and -4.0MPa, before and after fruitings, respectively. Excellent water providing capacity (higher ψ) is expected to well-matured cultures with a high density of mycelial colonization.

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