THE KOREAN JOURNAL OF MYCOLOGY
Vol. 23, No 1, p53-60 March 1995
Printed in S. KOREA

Standardization of Chemically Defined Medium for the Production of Mycelium and Basidiocarps in *Flammulina velutipes*

Chi-Hyeun Song[1]*, Chang-Ho Lee[1], Jang-Hyuk Ahn[2], Bum-Shik Hong[3] and Han-Chul Yang[2]

[1]**Dept. of Biotechnology, Daegu Univ., Kyungsan, Kyungbuk, 713-714*
[2]*Dept. of Food Engineering, Korea Univ., Seoul, 136-701*
[3]*Dept. of Food and Biotechnology, Korea Univ., Chochiwon, Chungnam, 339-800*

팽나무버섯 균사체 및 자실체 생산을 위한 화학합성배지의 최적화

송치현[1]* · 이창호[1] · 안장혁[2] · 홍범식[3] · 양한철[2]

[1]*대구대학교 생물공학과*
[2]*고려대학교 식품공학과*
[3]*고려대학교 식품생명공학과*

ABSTRACT: Nutritional requirements for the growth of *Flammulina velutipes* were studied. Mannitol, glutamic acid and ammonium nitrate were chosen for the maximum mycelial growth when various carbon and nitrogen sources tested. Optimum C : N ratio for the mycelial growth was 20 : 1. Potassium dihydrogen phosphate was selected among the phosphate sources. Magnesium sulphate and thiamine HCl stimulate mycelial growth. Final compositions of optimized chemically defined medium were 1.5% mannitol, 0.082% NH_4NO_3, 0.312% glutamic acid, 0.25% KH_2PO_4, 0.06% $MgSO_4 \cdot 7H_2O$ and 0.3 μg/l thiamin HCl. This medium not only support mycelial growth but also induce fruit body formation.

KEYWORDS: *Flammulina velutipes*, Nutritional requirements, Fungal development

Flammulina velutipes is an edible mushroom which is commonly known as winter mushroom because it appears most frequently in winter (Tonomura, 1978). It belongs taxonomically to the family of Tricholomataceae. The artificial cultivation was started since 1899 in Japan and it was forth in total world production of edible mushroom in 1984 (Chang and Miles, 1989). The consumption of this mushroom continues to increase due to it has high protein content, anti-cancer effect and lowering blood cholesterol level (Komatsu *et al.*, 1963, Lin *et al.*, 1974).

The life cycle of *F. velutipes* differs from other basidiomycetes since monokaryotic fruit bodies are formed and dedikaryotization is displayed. This mushroom is also well known for inducing a haploid fruit body on monokaryons (Ross, 1979). Physiologically, it is a chemoheterotroph which consumes organic carbons. Cellulose, lignin, and monosaccharides are major carbon sources for the saprophytic growth of this fungi and it is uncommon for parasitic growth (Tonomura, 1978). Amino acids and ammonium compounds are the principle nitrogen sources and Mg^{2+} and PO_4^{3-} are essential inorganic salts for the growth of mycelium and fruit body formation. Various metal ions and vitamins are also required (Chang and Miles, 1989). In nature, it breaks down the stem and

*Corresponding author

roots of broadleaved trees such as aspen, willow, mulberry, poplar and chinese nettle (Kitamoto, 1972).

Previously, additives for the stimulation of both mycelial and fruit body growth and cultivation medium by using agricultural waste were developed in our laboratory (Song *et al.*, 1993, Ahn *et al.*, 1993). In the present study, a chemically defined medium was developed which can support both mycelial and fruit body growth of *F. velutipes* in order to study systematically on fungal physiology and development.

Materials and Methods

Source of culture and medium

Flammulina velutipes (FV 10) was obtained from Dr. K. Y. Cho, the university of sydney, and was maintained and cultured on Henneberg's medium (glucose 5.0%, KNO_3 0.2%, $NH_4H_2PO_4$ 0.1%, KH_2PO_4 0.1%, $CaCl_2$ 0.01%, $MgSO_4 \cdot 7H_2O$ 0.05%) (Wakita, 1955).

Preparation of inoculum

Mycelium of *F. velutipes* was grown on Henneberg's medium in petri dish. Culture on the plate was punched by 8 mm diameter with sterilized cork boler. The agar disc containing hyphal tip was used as inoculum for the solid culture. For the submerged mycelial culture, mycelial pellets obtained by growing medium in shake cultures in 250 m*l* flask containing 100 m*l* of Henneberg's medium (pH 6.5, 22℃, 125 rpm, 15 days) were homogenized aseptically with glass beads (3 mm dia.) by shaking. One ml of mycelial suspension (3.0 mg/m*l*, D.W.) was used for inoculation.

Formulation of chemically defined medium

F. velutipes was grown in Henneberg's agar medium modified as follows : different source of carbohydrates, inorganic nitrogen and amino acids were used to substitute the nutrient sources in the original Henneberg's medium as detailed in Table 1 and Table 2. The C:N ratio was alterd by keeping the carbon constant and varying the level of nitrogen to obtain ratios from 1 : 1 to 200 : 1. Petri dishes (80 mm dia.) containing 15 m*l* each of the variously modified medium at pH 6.5 were inoculated with mycelial disc on the center of the plate and the colony diameter was measured after 8 days.

The carbohydrate, inorganic nitrogen, amino acid and C : N ratio supporting maximum mycelial growth of *F. velutipes* were chosen for further study. Different concentration of maltose, inorganic salt, trace metal and thiamin were also tested to determine the optimum concentrations of these ingredients required for mycelial growth and basidiocarp formation. Environmental conditions were varied in order to obtain optimum cultural characteristics as follows : 1) Temperature ; The inoculated flasks were incubated at 5, 10, 15, 20, 25 and 30℃. 2) pH ; The medium was adjusted to different level of pH with 0.1 M HCl or NaOH. The final pH before autoclaving were 2, 3, 4, 5, 6, 7 and 8. 3) Shaking frequencies ; Flasks were shaken at 40, 60, 80, 100, 120, 140, 160, 180 and 200 rpm.

Measurement of mycelial growth

Diameter of mycelial colony was measured on the day 8 after inoculation with a mycelial disc on the center of the plate for the solid culture and expressed as mm. In the submerged culture, mycelium consisting of individual pellets was harvested by filtration (Whatman NO. 42) and washed 3 times with distilled water on the day 15 after inoculation with 1 m*l* of precultured mycelial suspension as mentioned above. Dry cell weight (DCW) of mycelium was measured by constant weight method at 105℃ and expressed as mg/m*l*.

Conditions for the basidiocarp formation on the chemically defined medium

Petri dish containing mycelium was incubated at 20~22℃ for 28 days under dark condition after inoculation. This plate was then incubated at 10~12℃ for 15 days for primordium formation. Finally, basidiocarps can be obtained after 10 days at 4~6℃ under 200 Lux light.

Table 1. Mycelial growth of *Flammulina velutipes* on Henneberg's medium with different source of carbon and nitrogen.

Carbohydrates (0.1 M)	Dia. of colony (cm)	Inorganic nitrogen sources (0.05 M)	Dia. of colony (cm)	Organic nitrogen sources (0.02 M)	Dia. of colony (cm)
Mannitol	6.1± 0.2	Ammonium nitrate	5.8± 0.3	Glutamic acids	6.7± 3
Rhamnose	5.7± 0.3	Ammonium sulfate	5.7± 0.3	Alanine	6.5± 2
Xylose	5.6± 0.2	Ammonium bicarbonate	5.7± 0.2	Valine	6.3± 2
Sorbotol	5.5± 0.1	Sodium nitrate	5.4± 0.3	Glycine	6.2± 3
Lactose	5.4± 0.2	Ammonium phosphate	5.4± 0.1	Asparagine	6.2± 1
CMC[1)]	5.2± 0.3	Ammonium chloride	5.3± 0.2	Isoleucine	6.0± 2
Ribitol	5.2± 0.2	Potassium nitrate	5.1± 0.2	Proline	5.7± 3
Glucose	5.1± 0.2	Ammonium tartrate	4.1± 0.3	Aspartic acid	5.5± 3
Mannose	5.0± 0.3	Ammonium acetate	2.4± 0.4	Arginine	5.5± 2
Inositol	4.9± 0.2	Ammonium citrate	2.0± 0.3	Serine	5.4± 2
Fructose	4.9± 0.2	Ammonium oxlate	0	Phenylalanine	5.3± 2
Starch[1)]	4.8± 0.1	Ammonium fosrmate	0	Trytophan	5.0± 3
Arabinose	4.7± 0.3	Ammonium thiocynate	0	Leucine	5.0± 2
Maltose	4.7± 0.2	Potassium nitrate	0	Threonine	5.0± 3
Dextrin[1)]	4.6± 0.3	Sodium nitrate	0	Methionine	4.5± 2
Ribose	4.3± 0.3			Cysteine	4.2± 3
Galactose	3.0± 0.2			Histidine	4.2± 3
Sucrose	2.0± 0.4			Lytsine	3.4± 4

[1)] Same weight of glucose

Results and Discussion

Nutritional requirements for the mycelial growth

F. velutipes can utilize a wide variety of carbohydrates such as mono-, di- and polysaccharides. Generally monosaccharides were better carbon source than di- and polysaccharides for the mycelial growth of *F. velutipes* (Table 1). Especially, mannitol achived the maximum mycelial growth among the carbon sources tested and this result agrees with Gruen and Wu (1972). On the other hand, Kitamoto (1972) found that trehalose was the best carbon source for the formation of fruit body. It may be due to the difference of nutritional requirement for the developmental stages of fruit body. *F. velutipes* can utilize polysaccharide such as starch, carboxymethyl cellulose (CMC) and dextrin. This result reveals that mycelium of this fungi can excrete both extracellular amylase and cellulase. The relatively poor growth of *F. velutipes* in sucrose and galactose could be due to the deficiency of enzyme which can decompose of this substrates.

Among the inorganic nitrogen sources tested, maximum mycelial growth was observed in ammonium nitrate (Table 1). The failure of growth in the medium containing sodium nitrite and potassium nitrite resembles a common situation in fungi (Garraway and Evans, 1984). The toxicity of nitrite could be due to its ability to deaminate amino acid and interfere with sulphur metabolism since its similarity to the sulphite ion (Pateman and Kinghorn, 1976).

Glutamic acid was the most effective amino acid for the mycelial growth from 18 kinds of amino

Table 2. Determination of optimum C : N ratio for the mycelial growth of *Flammulina velutipes.*

C : N ratio[1)]	Diameter of mycelial colony (cm)
1 : 1	3.6± 0.2[2)]
5 : 1	5.8± 0.1
10 : 1	5.8± 0.2
15 : 1	6.1± 0.1
20 : 1	6.2± 0.3
40 : 1	5.8± 0.2
60 : 1	5.8± 0.3
80 : 1	5.9± 0.3
100 : 1	6.0± 0.3
120 : 1	5.8± 0.2
140 : 1	6.4± 0.1
160 : 1	6.4± 0.1
180 : 1	6.5± 0.1
200 : 1	5.5± 0.2

[1)] C: Mannitol 1.5% (W/V) as a carbon source
N: Ammonium nitrate as a nitrogen source
[2)] ± SD: Standard Deviation, 5 replicates
Culture was carried out at 22℃ for 8 days

acids tested (Table 1). Simple structural amino acids such as glycine, alanine and valine were also effective for the mycelial growth of *F. velutipes.* When glutamic acid was not added to the submerged culture, the growth of mycelium was inhibited fatally and ceased.

F. velutipes grew wide range of C : N ratio (Table 2). Mycelial growth of this fungi was good at C : N ratio of 5 : 1−80 : 1 and was not significantly different at such ranges. The best yield was obtained in the C : N ratio of 20 : 1 which equals to 1.5% (W/V) of mannitol and 0.085% (W/V) of ammonium nitrate. Similar results were reported from other kinds of fungi. For instance, optimum C : N ratio of Volvariella volvacea (Chang-Ho and Yee, 1977) and *Lentinus edodes* (Song *et al.*, 1987) was obtained 20 : 1 and 30 : 1, respectively.

Phosphate sources of ammonium form were not effective to the mycelial growth of *F. velutipes* while sodium or potassium form promoted mycelial growth (Fig 1). Garraway and Evans (1984) reported that phosporus deficiency in the liquid culture caused greater inhibition in growth than any other mineral deficiency. The optimum concentration of potassium dihydrogen phosphate for the mycelial growth was 0.25% (W/V) (Fig. 2).

There was no stimulatory effect on the mycelial

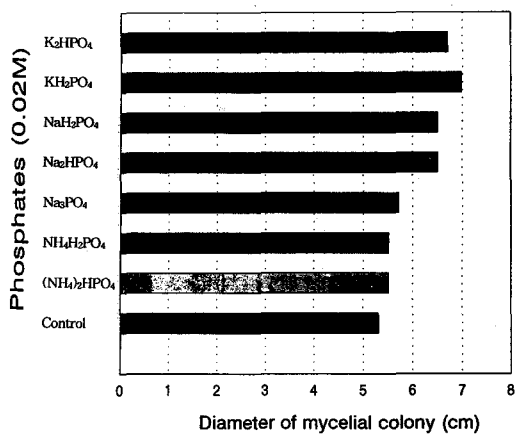

Fig. 1. Mycelial growth of *Flammulina velutipes* on the basal medium with various sources of phosphate.
Mycelial compactness: ■: compact, ▩: thin.
Culture was carried out at 22℃ for 8 days.

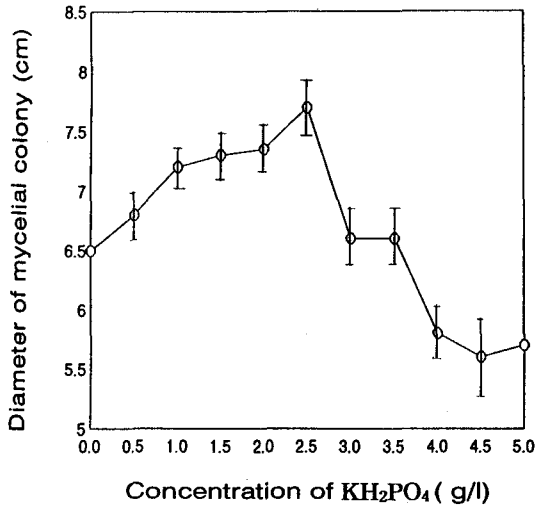

Fig. 2. Effect of potassium dihydrogen phosphate concentration on the mycelial growth of *Flammulina velutipes.*
Culture was carried out at 22℃ for 8 days.

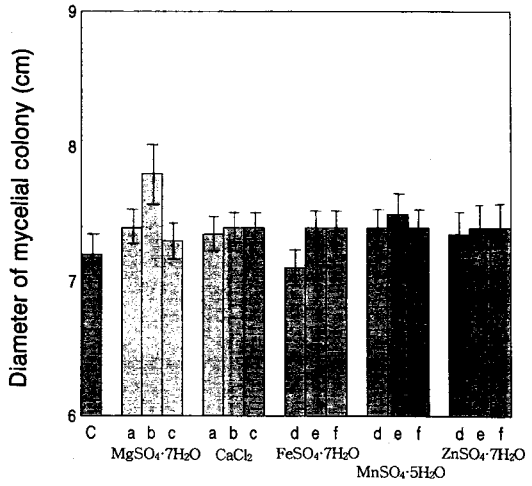

Fig. 3. Mycelial growth of *Flammulina velutipes* on the basal medium with various minerals.
Concentration (g/*l*): C (control) (a) (f) 0.1, (b) 0.001, (c) 1.0 (d) 0.01, (e) 0.05.
Culture was carried out at 22℃ for 8 days.

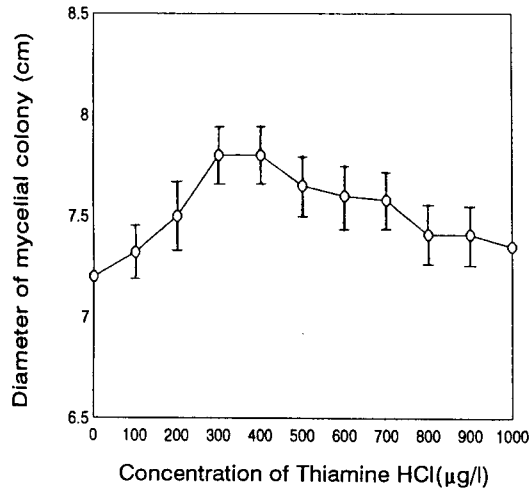

Fig. 4. Effect of thiamine hydrochloride concentration on the mycelial growth of *Flammulina velutipes.*
Culture was carried out at 22℃ for 8 days.

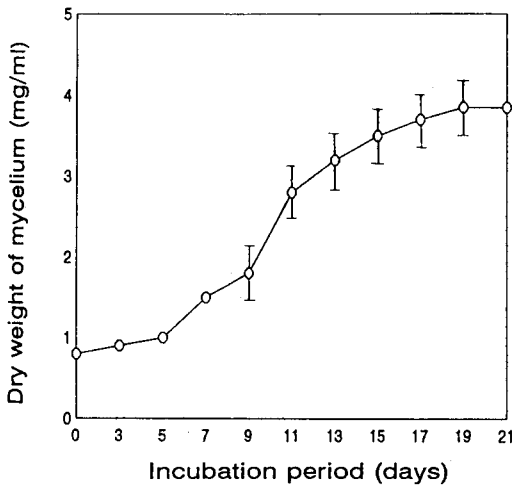

Fig. 5. Mycelial growth of *Flammulina velutipes* in the optimized chemically defined medium by submerged culture.
Culture was carried out at 22℃ for 21 days.
Composition of optimized chemically defined medium (g/*l*).
Mannitol: 15, NH_4NO_3: 0.82, Glutamic acid: 3.12, KH_2PO_4: 2.5, Thiamine HCl: 0.3 (ug/*l*), $MgSO_4 \cdot 7H_2O$: 0.06

growth by addition of minerals except $MgSO_4$ (Fig. 3). It is generally known that Mg^{2+} is necessary for cofactor of enzymes related in glycolysis and TCA cycle.

The mixture of various water soluble vitamins was added to the Henneberg's medium to investigate the requirements of vitamins. Mycelium did not grow well in the thiamine deficient medium but it was hard to distinguish the difference of growth on the medium containing various vitamin mixtures (data not presented). Optimum concentration of thiamine HCl was observed at 300 ug/*l* (Fig. 4). However, Hong (1981) reported that the maximum mycelial growth was obtained in the concentration of 500 ug/*l* and decrease in over that concentration.

Finally, composition and optimum concentration of chemically defined medium was standardized and maximum mycelial yield was obtained after 19 days in the submerged mycelial culture (Fig. 5)

Environmental conditions for the mycelial growth in liquid culture

Optimum temperature for the mycelial growth was observed at 20℃ (Fig. 6). There was no significant difference in growth at 25℃. However, the growth was decreased over 25℃. Similar result

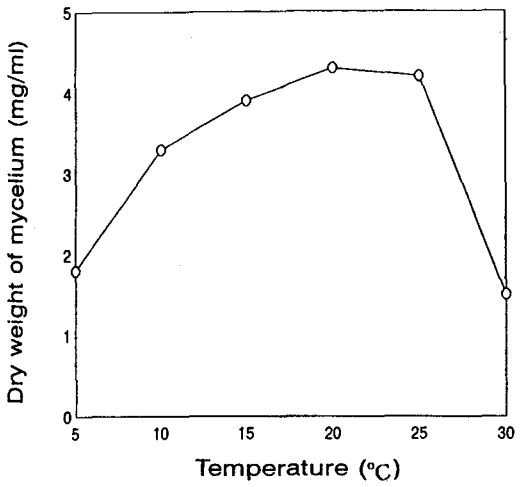

Fig. 6. Effect of temperature on the mycelial growth of *Flammulina velutipes* in submerged culture.
Culture was carried out with reciprocal shaker at 100 rpm for 15 days.

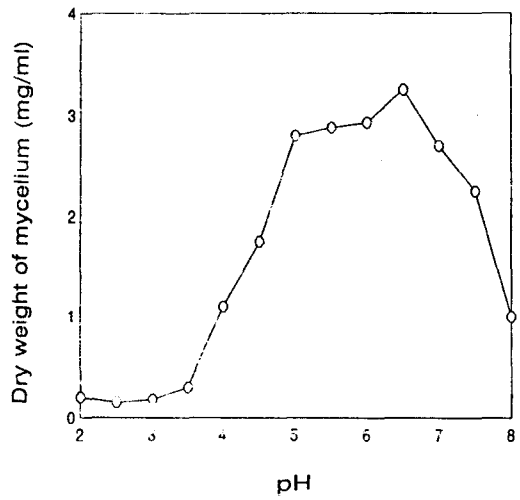

Fig. 7. Effect of pH on the mycelial growth of *Flammulina velutipes* in submerged culture.
Culture was carried out at 22℃ for 15 days.

was reported that mycelial growth of *F. velutipes* was ceased at over 34℃ for a short period of time (Tonomura, 1978).

Different levels of pH exerted significant effect on mycelial growth of *F. velutipes* (Fig. 7). Optimum pH was 6.5. In comparison to other edible fungi, this fungi required a relatively high pH.

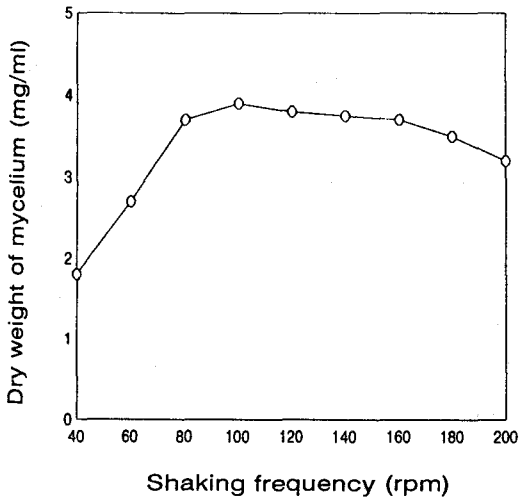

Fig. 8. Effect of shaking frequency on the mycelial growth of *Flammulina velutipes* in submerged culture.
Culture was carried out at 22℃ for 15 days.

For instance, Optimum pH for *Lentinus* and *Pleurotus* sp. was 4.5~5.0 (Song *et al.*, 1987).

Maximum mycelial growth was obtained with 100~120 rpm of rotation speed (Fig. 8). Shaking frequency affected not only oxygen tension but also size of pellet. Yoshida *et al.* (1965) observed that the growth rate of *L. edodes* was greatly affected by oxygen tension as is case for cultures of other filamentous fungi since the rate of respiration of mycelial pellets decreased with the increasing pellet diameter. In his study on the respiration characteristics of mycelia, he indicated that the aspect of oxygen utilization by mycelial mass changed on accordance with the variation of growth type (filamentous or pellet). It could be proposed that pellets did not continue to grow exponentially after they attained a certain diameter and eventually a pellet was made up of peripheral growing zone and nongrowing inner mass which lack of oxygen.

Basidiocarp formation from the chemically defined medium

Irrespective of natural habitat or artificial culture as regard to fungal growth, there are two different growth stages, i.e., vegetative growth

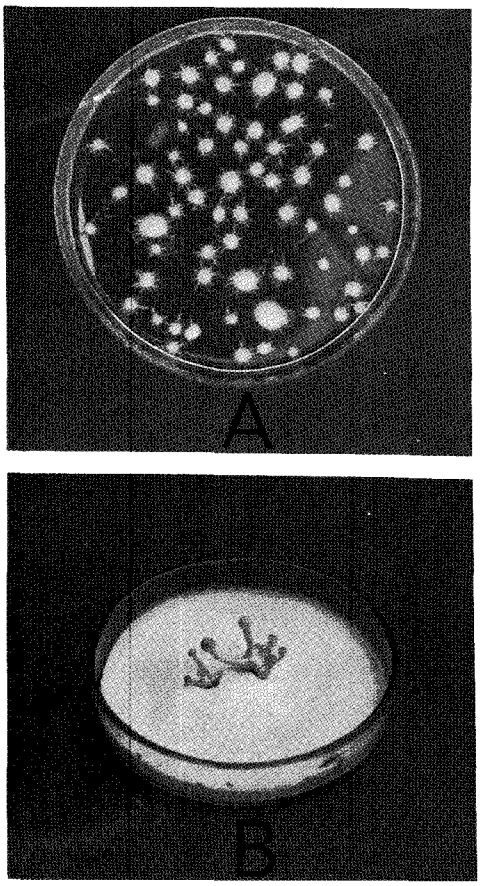

Fig. 9. Mycelial pellets (A) and basidiocarps (B) of *Flammulina velutipes* in the optimized chemically defined medium.

phase, namely mycelial development, and the reproductive growth phase in which mushroom grows up and produces spores. However, only fragment knowledge exists concerning the physiology and biochemistry of fruitiy of *F. velutipes*. In part, this is due to the difficulty of fruiting edible fungi in pure culture on chemically defined media. Our result has shown that the optimized chemically lefined medium not only supported mycelial gro-th but also induced fruiting body formation (Fig. This result can be applicable for the study of ysiology, biochemistry and fructification of *F. utipes*.

적 요

mmulina velutipes(팽나무버섯)의 영양요구성에 대한 연구로써 각종 탄소원 및 질소원을 처리한 결과 mannitol과 glutamic acid 그리고 ammonium nitrate가 최대 균사체 생장을 보였으며, 최적 C : N ratio 로써 20 : 1이 선정되었다. Phosphate source 로는 potassium dihydrogen phosphate가 좋은 결과를 나타내었으며, magnesium sulphate와 thiamin HCl이 균사체 생장을 촉진하였다. 이상의 결과로 최적 화학합성배지의 조성은 1.5% mannitol, 0.082% NH_4NO_3, 0.312% glutamic acid, 0.25% KH_2PO_4, 0.06% $MgSO_4 \cdot 7H_2O$ 그리고 0.3 ug/*l* thiamin HCl 로 나타났다. 이와 같은 배지의 조성은 균사체 생장 뿐만 아니라 자실체 생장에도 효과가 있는 것으로 나타났다.

References

Ahn, J. H., Lin, W. J., Song C. H. and Yang, H. C. 1993. *Kor. J. Appl. Microbiol. Biotechnol.* **21**(4): 306-309.

Chang-Ho, Y. and Yee, N. T. 1977. Comparative study of the physiology of *Volvariella volvacea* and *Coprinus cinereus*. *Trans. Br. Mycol. Soc.* **68**: 167-172.

Chang, S. T. and Miles, P. G. 1989. Mushroom science in "Edible Mushrooms and their cultivation". CRC Press, Inc., pp. 163-170.

Garraway, M. O. and Evans, R. C. 1984. Nutrition as a basis for the study of fungi in "Fungal nutrition and phisiology" John Wiley and Sons Inc. U.S. A, 1-21, 71-221.

Gruen, H. E. and Wu, S. H. 1972. Promotion of stipe elongation in isolated *Flammulina velutipes* fruit bodies by a carbohydrates, natural extracts and amino acid. *Can. J. Bot* **50**: 803-818.

Hong, J. S. and Yoon, S. 1981. Fruit-body formation of *Flammulina velutipes* on the synthetic medium. *Korean. J. Food Sci. Technol., 13, No. 3.*

Kitamoto, Y. 1972. Mechnism of fruiting Mushrooms (Tokyo). **4**: 9-18.

Komatsu, J., Terekawa, H., Nakanishi, K. and Watanabe, Y. 1963. *Flammulina velutipes* with antitumor activities. *J. Antibiot., Ser. A.* **16**: 139-143.

Lin, J. Y., Lin, Y. J., Chen, C. C., Wu, H. L., Shi, G. Y. and Jeng, T. W., 1974. Cardiotoxic protein from edible mushrooms. *Nature (London).* **252**: 235-237.

Pateman, J. A. and Kinghorn, J. R. 1976 Chapter 7. Nitrogen metabolism in "The Filamentous fungi" Eds. Smith, J. E. and D. R. Berry. John wiley and sons. N. Y. Vol. 2. pp. 159-237.

Ross. I. K. 1979. Biology of the fungi. McGraw-Hill

Co. pp. 140-177.

Song. C. H., Cho, K. Y., and Nair, N. G. 1987. A synthetic medium for the production of submerged cultures of *Lentinus edodes. Mycologia,* **79**(6): 866-876.

Song. C. H., Lee, C. H., Huh, T. L., Ahn, J. H. and Yang, H. C. 1993. Development of substrates for the production of basidiocarps of *Flammulina velutipes. The Korean J. Mycology.* **21**(3): 212-216.

Tonomura, H. 1978. *Flummulina velutipes* in "The biology and cultivation of edible mushrooms". Eds. Chang, S. T. and Hayes, W. A. Academic Press pp. 410-421.

Wakita, S. 1955. Biochemical studies on the velvet-mushroom, *Collybia velutipes.* Part 3. Effect of amino acids and vitamines in the growth of the mycelium. *Agr. Chem. Soc. Japan.* **28**(9): 707-711.

Yoshida, T., Taguchi, H. and Teramoto, S. 1965. Studies on submerged culture of basidiomycetes. 1. Some factors affecting the growth of shiitake (*Lentinus edodes*). *J. Ferment. Technol.* **43**: 325-334.