J. Trop. Agric. and Fd. Sc. 35(1)(2007): 131–138

Optimization of submerged culture for the production of *Lentinula edodes* mycelia biomass and amino acid composition by different temperatures

(Pengoptimuman kultur ampaian dalam penghasilan biojisim miselium *Lentinula edodes* dan komposisi asid amino pada suhu berlainan)

H. Aminuddin*, A. Mohd Khan**, H. Abidin*, K. Madzlan***, R. Suri*** and M.K. Kamal***

Key words: *Lentinula edodes*, mycelium, submerged culture, amino acid, temperature

Abstract

Lentinula edodes and its mycelium are known for their unique properties and have been used in food and medicinal industry for centuries. The effect of different temperatures and medium conditions on yield of dry mycelium and total amino acid content was studied. Submerged culture technique was employed to grow mycelium in PDB (potato dextrose broth) and PDB++ (PDB, yeast extract, malt extract, peptone, KH_2PO_4 and $MgSO_4.7H_2O$) media under different temperatures. The amino acid composition of the mycelium was determined by the High Performance Liquid Chromatography techniques (HPLC). An optimum growth of the mycelium was observed on the PDB++ medium incubated at 25 °C. The mycelium harvested had the highest content of amino acid, of which the major component was glutamic acid.

Introduction

Lentinula edodes belongs to the class of Basidiomycetes and the family of Tricholomataceae, which is under the order of Agaricales. This mushroom *(Plate 1)* is the second most popular edible mushroom worldwide and has delicious flavour, great nutritional value and immunity-enhancing components (Jong and Birmingham 1993). It is also named "Elixir of Life" in China and Japan (Rudic and Dronina 2001).

Plant and food scientists are seeking production improvements from existing sources and attempting to develop new

Plate 1. ***Lentinula edodes*** *(shiitake) mushroom*

*Biotechnology Research Centre, MARDI Headquarters, Serdang, P.O. Box 12301, 50774 Kuala Lumpur, Malaysia
**School of Chemical Sciences and Food Technology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia
***Food Technology Research Centre, MARDI Headquarters, Serdang, P.O. Box 12301, 50774 Kuala Lumpur, Malaysia
Authors' full names: Aminuddin Hussin, Mohd Khan Ayob, Abidin Hamid, Madzlan Kasran, Suri Roowi and Mohamad Kamal Abd. Kadir
E-mail: aminh@mardi.my

sources of protein. Moulds, like other microorganisms, have the potential to produce large amounts of protein depending on genera and species selected and the cultural conditions used (Donald et al. 1976). Cultural factors such as medium components and temperature are known to influence microbial growth. Although the effect of temperature on the growth of moulds depends to some extent on the organism used, the range of 22 °C to 26 °C seems to give the best yields of mycelium (Ganisan 2001).

Industrial bioprocesses using filamentous fungi encompass the production of majority of commercially important products with regard to the quantity as well as the diversity of metabolites. The production is mainly via the submerged culture processes, where a dynamic relationship exists between environmental conditions and the growth pattern of these modular microorganisms. Mycelia of a fungus may be cultured on a variety of substrates/media, either liquid or solid (by the addition of agar). Submerged culture may have potential advantages of higher production of mycelia in a compact space and shorter time with lesser chances for contamination (Friel and McLoughlin 2000). It is also used to produce products supplying a large fraction of the world's pharmaceutical and biotechnology market (Arora et al. 1992).

The objectives of this study were to identify the effect of different temperatures on the growth of mycelium and also to determine amino acid composition of the mycelium.

Materials and methods

Mushroom *(Lentinula edodes)* sample was purchased from Vita Agrotech Sdn. Bhd. Spores from the mushroom fruiting bodies were collected and inoculated on potato dextrose agar (PDA) media. The inoculated media were incubated in an oven at 25 °C for 14 days until growth of mycelium was observed. Mycelium was then isolated from fruiting body and maintained as slope on the PDA at 25 °C for 1 week (dark condition) and stored at 4 °C as stock culture. Mycelium from the stock culture was inoculated on fresh PDA at 25 °C for 1 week and stored at 4 °C in dark condition.

Growth media

A standard medium, potato dextrose broth (PDB: 24 g, pH 6) and a modified PDB (PDB++) were employed in this study. The modified medium was as described elsewhere (Aminuddin et al. 2003) which consisted of PDB 20 g, yeast extract 5 g, malt extract 5 g, peptone 5 g, potassium phosphate 2 g and magnesium sulfate heptahydrate 0.5 g and adjusted to pH 6.

Inoculation procedure

Two-week-old mycelium agar disc (9 mm diameter) of PDA plates was inoculated into a 250 ml conical flask containing 25 ml broth (PDB or PDB++) and incubated for 30 days at 25 °C in the dark condition *(Plate 2)*. The pH of the broth was adjusted to 6 with 1 N NaOH to avoid possible inhibition of growth of mycelium due to acidic condition.

Determination of biomass

Growth of mycelium was evaluated based on the dry weight of harvested mycelia. Harvesting was carried out by filtering the growth media with filter paper (Whatman No. 5) followed by washing with excess

Plate 2. Suspension culture

amount of distilled water. Then the mycelium was freeze-dried for 5–8 h (Labconco 195, England) to a constant weight. The growth of mycelium was measured for every three days.

$$\text{Specific growth rate (g/ml/d)} = \frac{(A1 - A2)g}{\dfrac{\text{total volume of sampling (ml)}}{\text{total time of sampling (d)}}}$$

Where: A1 = sample weight before freeze-dried
A2 = sample weight after freeze-dried

Amino acid composition

Hydrolysis Individual amino acids were determined after digestion of samples in 6 N HCl at 110 °C as described by Blackburn (1968). The sample was hydrolysed in triplicates using the sealed-tube hydrolysis method. About 0.05–0.1 g of the homogenized sample was weighed into a test tube. Sample of 10 ml of 6 N HCl was added into the tube before flushing with nitrogen. The tube was then placed in an oven at 110 °C for 24 h for hydrolysis of samples. After hydrolysis, the sealed end of the tube was opened and cooled. The hydrolysate was then transferred into a 100 ml volumetric flask, and 400 µl of 50 µmole/ml of alpha amino butyric acid (AABA) was added before being made up to volume with deionised water. The hydolysate was then double filtered through filter paper (Whatman No. 541).

Derivatization Derivatisation was carried out according to Cohen and De Antonis (1994) using borate buffer and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AccQ-tag reagent). A volume of 70 µl of borate buffer and 10 µl of sample were mixed and vortexed to increase the pH of the sample. Then 20 µl of AccQ-tag reagent was added and vortexed immediately. The sample was kept at room temperature for 1 min to complete the derivatization process.

Amino acid analysis

The amino acid content of samples either as 6 N HCl or performic acid hydrolysate was analysed and quantitatively determined using Waters 510 HPLC system (Waters, USA) and detected by Waters 470 fluorescence detector at E_λ and E_m of 250 nm and 395 nm. Then 10 µl of the derivatised sample was injected in gradient mode into the HPLC column and the amino acids were eluted automatically. The column used was Waters AccQ-Tag Amino Acid Analysis Column (3.9 mm id x 150 mm) at 36 °C.

Mobile phase A was prepared by diluting 200 ml of concentrated AccQ-Tag Eluent A with 2,000 ml of distilled water. Mobile phase B was 60% acetonitrile and the flow rate was 1.0 ml/min. The quantity of each amino acid eluted was determined based on the chromatogram recorded by the machine. The calculation for each amino acid is as follows:

Calculation of response factor, Rf:

$$Rf = \frac{W_{is} \times A_{std}}{W_{std} \times A_{is}}$$

Where: Rf = Standard response factor
W_{is} = Wt. of internal standard, AABA
A_{is} = Peak area of internal standard
W_{std} = Wt. of standard
A_{std} = Peak area of standard

$$\text{Amino acids content (g/100 g sample)} = \frac{A_s \times W_{is} \times 100}{A_{is} \times W_s \times Rf}$$

where: A_s = Peak area of sample
A_{is} = Peak area of internal standard
W_s = Wt. of sample (g)
W_{is} = Wt. of internal standard
Rf = Response factor

Results and discussion

In natural environment, fungi can be exposed to a wide range of temperatures, with daily and seasonal variations, so active growth may not always be possible. Two media, PDB (potato dextrose broth)

and PDB++ (enriched potato dextrose broth) were tested for the cultivation of mycelium of *L. edodes*. To find the optimal temperature for growth of mycelium and amino acid production, this mycelium was cultivated at various temperatures (15, 20, 25, 30 °C). Proper aeration was also assured through sterile cotton plug of the cultivation flask.

An increase in biomass concentration was observed with increased temperature. The optimal temperature for mycelial growth in PDB submerged culture was at 25 °C which produced 0.0442 g/biomass after 21 days of incubation *(Figure 1)*. This was followed by 0.428 g/biomass (27 d) and 0.0423 g/biomass (27 d) at 20 °C and 15 °C respectively. However, there were not much differences between yield of mycelium obtained at 15 °C and 25 °C with yield obtained at 20 °C. The growth of mycelium in the PDB incubated at 30 °C was rather static at day 3 to 9 incubation, but slowly decreased until the 15th day. Due to the high temperature, the growth only lasted for about 9 days.

The effect of different temperature incubation on the growth of mycelium in PDB++ medium is shown in *Figure 2*. The optimum growth of mycelium in PDB++ was observed when the suspension culture was incubated at 25 °C for 27 days producing 0.085 ± 0.012 g/biomass. Ganisan (2001) reported that the optimum range of temperature for vegetative growth of *L. edodes* is 24 ± 2 °C. However, there were minimal difference (p >0.05) between yields of mycelium obtained at 15 °C and 20 °C, which produced 0.056 ± 0.017 g/biomass at day 24 and 0.056 ± 0.010 g/biomass at day 21.

Data obtained showed that, when temperature was too low (15 °C and 20 °C), the cell metabolism activity would stop and inhibit the cell growth (Kamal 2002) while a higher temperature (30 °C) recorded shorter growth period. The decrease in the growth rate may reflect either a distruption of metabolic regulations or death of cells. Temperature affects the growth parameters of lag time, specific growth rate and total yield differently (Michael et al. 2001).

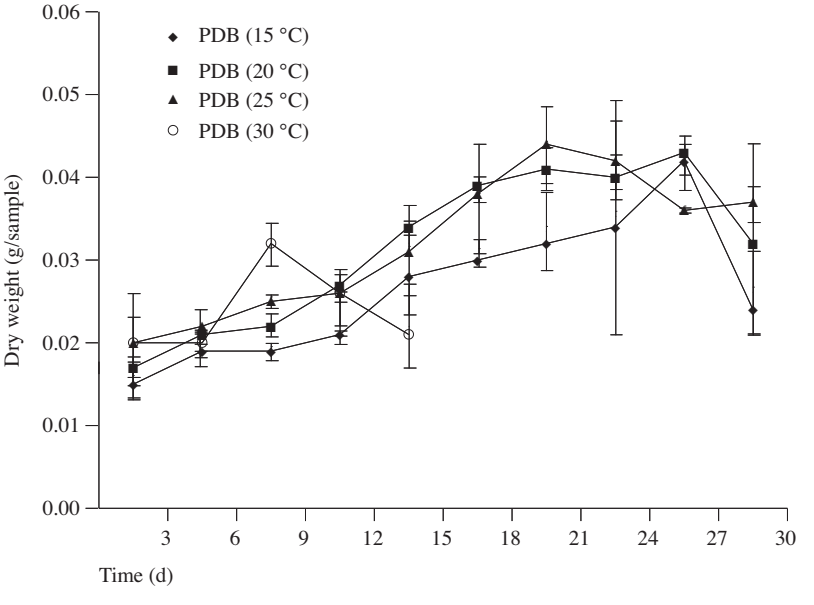

Figure 1. Effect of different temperatures on growth of mycelium in PDB submerged cultures of ***Lentinula edodes*** *mycelium. The error bars ± in the figure indicate the standard deviations from triplicate samples*

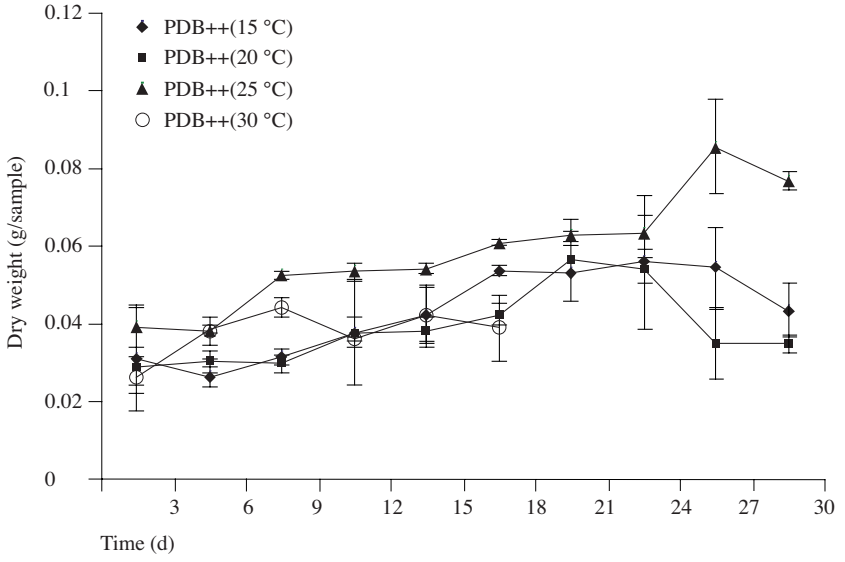

Figure 2. Effect of different temperatures on growth of mycelium in PDB++ submerged cultures of ***Lentinula edodes*** *mycelium. The error bars ± in the figure indicate the standard deviations from triplicate samples*

A significant difference was observed between yields obtained for both media. The growth of mycelium in PDB medium was relatively lower than PDB++ medium.

Most microorganisms require carbon and nitrogen sources or a mixture of amino acids and not just simple nitrogen sources to support the growth of mycelium. Sakamoto et al. (1978) reported that high concentrations of carbohydrate and nitrogen sources are usually needed in order to achieve a high yield (dry weight) of mycelium. But too high concentration of glucose can have an inhibitory growth effect on several mushrooms. Temperature and medium affect the rate of cell reactions, nutritional requirement, the nature of metabolism and also biomass composition.

The amounts for each amino acid are shown in *Table 1*. The HPLC analysis showed that the submerged culture of *L. edodes* mycelium contained 16 amino acids namely aspartic acid, serine, glutamic acid, glycine, histidine, arginine, threonine, alanine, proline, tyrosine, valine, methionine, lysine, isoleusine, leusine and phenylalanine. The most abundant of amino acids in both PDB and PDB++ cultures were glutamic acid followed by arginine. Mycelium in PDB++ incubated at 25 °C contained highest amount of amino acid ($p > 0.05$) compared to other parameters of temperature and medium. It produced 20.79 g/100 g sample of amino acid on the 27th day of incubation, followed by PDB++ 20 °C (13.39 g/100 g sample), PDB++ 30 °C (12.52 g/100 g sample), PDB++ 15 °C (11.45 g/100 g sample) and medium PDB with incubation at 25, 20, 30 and 15 °C respectively *(Table 2).*

This result implies that the highest yield of the mycelium not only varies with different carbon and nitrogen sources, but also with different temperatures. The amino acid composition is more important than crude protein content because of the significance of the amino acid profile to the potential nutritional value and due to the influence of the amino acid content on flavour.

Eight essential amino acids were found in mycelium for both PDB and PDB++ media such as histidine, threonine, valine, methionine, lysine, isoleucine, leucine and

Table 1. Specific growth rate on growth of mycelium in PDB and PDB++ submerged cultures of *Lentinula edodes* mycelium at different temperatures

Medium	15 °C	20 °C	25 °C	30 °C
PDB (g/ml/d)	6.27×10^{-5}	6.34×10^{-5}	8.42×10^{-5}	–
PDB++ (g/ml/d)	9.3×10^{-5}	1.07×10^{-4}	1.26×10^{-4}	–

PDB = Potato Dextrose Broth
PDB++ = Enriched Potato Dextrose Broth

Table 2. The composition of amino acid mycelium (g/100 g sample) with different temperatures in Potato Dextrose Broth (PDB) and Enriched Potato Dextrose Broth (PDB++) submerged cultures

Amino acid		15 °C	20 °C	25 °C	30 °C
Aspartic acid	(PDB)	0.42 ± 0.04e	0.66 ± 0.03d	1.16 ± 0.03b	0.48 ± 0.04e
	(PDB++)	1.01 ± 0.01c	1.11 ± 0.03b	2.12 ± 0.12a	0.96 ± 0.01c
Serine	(PDB)	0.25 ± 0.04f	0.42 ± 0.02d	0.69 ± 0.02b	0.32 ± 0.01e
	(PDB++)	0.56 ± 0.05c	0.59 ± 0.05c	1.04 ± 0.05a	0.59 ± 0.01c
Glutamic acid	(PDB)	0.92 ± 0.10e	1.28 ± 0.01d	2.02 ± 0.13c	0.86 ± 0.06e
	(PDB++)	2.12 ± 0.11bc	2.36 ± 0.03b	4.14 ± 0.42a	1.96 ± 0.03c
Glycine	(PDB)	0.25 ± 0.01f	0.41 ± 0.04de	0.47 ± 0.04d	0.31 ± 0.03ef
	(PDB++)	1.37 ± 0.10c	1.75 ± 0.09a	1.57 ± 0.05b	1.67 ± 0.06ab
Histidine	(PDB)	0.07 ± 0.01e	0.15 ± 0.01d	0.26 ± 0.02b	0.10 ± 0.01e
	(PDB++)	0.21 ± 0.03c	0.16 ± 0.01d	0.32 ± 0.04a	0.16 ± 0.01d
Arginine	(PDB)	0.40 ± 0.04e	0.64 ± 0.03d	0.88 ± 0.01c	0.45 ± 0.02e
	(PDB++)	0.66 ± 0.07d	0.95 ± 0.03b	1.47 ± 0.01a	0.88 ± 0.01c
Threonine	(PDB)	0.27 ± 0.04f	0.34 ± 0.03e	0.58 ± 0.02b	0.29 ± 0.01f
	(PDB++)	0.42 ± 0.03d	0.48 ± 0.01c	0.90 ± 0.02a	0.50 ± 0.02c
Alanine	(PDB)	0.36 ± 0.05d	0.73 ± 0.07c	0.82 ± 0.01bc	0.33 ± 0.03d
	(PDB++)	0.94 ± 0.03b	1.28 ± 0.10a	1.37 ± 0.02a	1.28 ± 0.25a
Proline	(PDB)	0.37 ± 0.03e	0.62 ± 0.01d	0.69 ± 0.02d	0.67 ± 0.05d
	(PDB++)	0.99 ± 0.04c	1.18 ± 0.01b	2.10 ± 0.15a	1.21 ± 0.01b
Tyrosine	(PDB)	0.14 ± 0.01d	0.16 ± 0.01d	0.32 ± 0.01b	0.14 ± 0.01d
	(PDB++)	0.23 ± 0.02c	0.21 ± 0.02c	0.51 ± 0.04a	0.21 ± 0.01c
Valine	(PDB)	0.31 ± 0.01e	0.44 ± 0.01d	0.62 ± 0.01c	0.34 ± 0.01e
	(PDB++)	0.63 ± 0.02c	0.68 ± 0.02b	0.99 ± 0.07a	0.67 ± 0.01bc
Methionine	(PDB)	0.05 ± 0.01e	0.07 ± 0.01cd	0.06 ± 0.01cde	0.05 ± 0.01de
	(PDB++)	0.12 ± 0.02b	0.11 ± 0.02b	0.21 ± 0.01a	0.07 ± 0.01c
Lysine	(PDB)	0.31 ± 0.01e	0.37 ± 0.03cde	0.90 ± 0.03a	0.33 ± 0.07de
	(PDB++)	0.45 ± 0.01cd	0.60 ± 0.02b	0.97 ± 0.17a	0.48 ± 0.05c
Isoleucine	(PDB)	0.26 ± 0.01e	0.39 ± 0.01d	0.66 ± 0.04b	0.30 ± 0.02e
	(PDB++)	0.51 ± 0.02c	0.57 ± 0.01c	0.94 ± 0.09a	0.55 ± 0.05c
Leucine	(PDB)	0.38 ± 0.01e	0.57 ± 0.01d	0.83 ± 0.01b	0.41 ± 0.01e
	(PDB++)	0.74 ± 0.01c	0.84 ± 0.01b	1.20 ± 0.07a	0.81 ± 0.02b
Phenylalanine	(PDB)	0.24 ± 0.01e	0.37 ± 0.02d	0.61 ± 0.01b	0.25 ± 0.03e
	(PDB++)	0.49 ± 0.01c	0.52 ± 0.01c	0.94 ± 0.04a	0.52 ± 0.01c

Mean values in the same column with same letters are not significantly different ($p > 0.05$)
The error bars ± in the table indicate the standard deviations from triplicate samples

phenylalanine. Essential amino acids in PDB mycelium suspension culture represent 1.89 to 4.52 g/100 g sample of the total amino acids, while mycelium in PDB++ contained 3.57 to 6.47 g/100 g sample. Results showed that, mycelium in PDB++ incubated at 25 °C contained highest essential amino acid compared to other parameters. The relative amount of essential amino acids varied depending on the medium.

The components of media, such as mineral salts and inorganic salts, influenced the flavour and amino acid composition of mycelium obtained. Based on the analysis of amino acids in mushrooms and mushroom mycelium, which showed an adequate level of all but the sulfur-containing amino acids, it seems that mushroom mycelium can be expected to be beneficial as a food supplement for human or animal consumption in the same way as other microbial protein sources (SCP) (Goldberg and Williams 1991).

Conclusion

Temperature affects the rate of cell reactions, the nature of metabolism, the nutritional requirement and biomass composition. The optimum temperature for growth of mycelium and highest production of amino acid was at 25 °C grown in PDB++ medium. Growth at 30 °C was short lived meanwhile growth of mycelium and amino acid composition at 15 °C and 20 °C were more moderate when PDB or PDB++ medium was used. A total of 16 amino acids were identified in mycelium submerged culture. The most abundant amino acid in mycelium culture was the glutamic acid. The appropriate harvest time, nitrogen and carbon sources, as well as aeration and mass transfer are critical factors in optimizing the production of mycelium and its amino acid composition in submerged culture.

Acknowledgement

The authors would like to thank all who have directly or indirectly contributed in the implementation of the project. This work was funded by IRPA (Research Grant No. 1900301634).

References

Aminuddin, H., Khan, M.A., Abidin, H., Kamal, M.K., Suri, R. and Madzlan, K. (2003). Mushroom Shiitake *(Lentinula edodes)* – mycelium culture. *Proceedings of Malaysian Science and Technology Congress 2003* (MSTC 2003), p. 285, Kuala Lumpur

Arora, D.K., Elander, R.P. and Mukerji, K.G. (1992). Introduction. In: *Handbook of applied mycologyy*. Vol. 4. (Arora, D.K., Elander, R.P. and Mukerji, K.G., eds.) p. 211–42. New York: Marcel Dekker

Blackburn, S. (1968). *Amino Acids Determination Methods and Techniques*. New York: Marcel Dekker

Cohen, S.A. and De Antonis, K.M. (1994). Application of amino acid derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. Analysis of feed grains, intravenous solutions and glycoproteins. *Journal of Chromatography 661:* 25–34

Donald, C.W.G., Keith, H.S. and Ross Hackler, L. (1976). Factors affecting production of mold mycelium and protein in synthetic media. *Appied and Environmental Microbiology 32(3):* 381–7

Friel, M.T. and McLoughlin, A.J. (2000). Production of a liquid inoculum/spawn of *Agaricus bisporus. Biotechnology Letters 22:* 351–4

Ganisan, K. (2001). Production biotechnology with controlled environmental regimes, substrates and enzyme markers for the Shiitake mushroom, *Lentinula edodes*. Master Science Thesis, Universiti Putra Malaysia, Serdang, Selangor

Goldberg, I. and Williams, R. (1991). *Biotechnology and Food Ingredients*. New York: Van Nostrand Reinhold

Jong, S.C. and Birmingham, J.M. (1993). Medicinal and therapeutic value of the Shiitake mushroom. *Advances in Applied Microbiology 39:* 153–84

Kamal, M.K. (2002). Penghasilan Goniotalamin daripada kultur ampaian *Goniothalamus umbrosus*. Master Science Thesis, Universiti Kebangsaan Malaysia, Bangi, Selangor, Malaysia

Michael, J.C., Sarah, C.W. and Graham, W.G. (2001). *The Fungi*. 2nd ed. New York: Academic Press

Rudic, V. and Dronina, A. (2001). Deep culturing of the *Lentinus Genus* (Berk) Sing 370 edible mushrooms on Lignocellulose substrate. *Romanian Biotechnological Letters 6(5):* 423–7

Sakamoto, R., Niimi, T. and Takahashii, S. (1978). Studies on submerged culture of edible fungi. I. Effect of carbon and nitrogen sources on submerged culture of edible mushroom. II. Submerged culture of edible fungi in high consistency starch media. *Journal of the Agricultural Chemistry Society of Japan 52(2):* 75–90

Abstrak

Keunikan *Lentinula edodes* dan miseliumnya telah diketahui umum dan telah digunakan dalam industri makanan dan perubatan sejak berabad lagi. Kajian dilakukan untuk melihat kesan suhu yang berbeza terhadap biojisim miselium kering dan kandungan jumlah asid amino. Teknik kultur ampaian telah digunakan untuk menumbuhkan miselium dalam PDB (kaldu dekstrosa kentang), dan PDB++ (PDB, ekstrak yis dan malta, pepton, KH_2PO_4 dan $MgSO_4.7H_2O$) pada suhu yang berbeza. Komposisi asid amino miselium ditentukan menerusi teknik Kromatografi Cecair Prestasi Tinggi (HPLC). Medium PDB++ dengan suhu 25 °C menghasilkan pertumbuhan miselium yang optimum dan miselium yang dituai mengandungi komposisi asid amino yang tertinggi, dengan asid glutamik merupakan asid amino yang utama.

Accepted for publication on 9 February 2006