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Effects of pH on mycelial growth and amino acid composition of *Lentinula edodes* in submerged cultures

(Kesan pH terhadap pertumbuhan miselium dan komposisi asid amino kultur terendam *Lentinula edodes*)

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Keywords: *Lentinula edodes,* mycelial, submerged culture, amino acid, pH

Abstract

Lentinula edodes (Shiitake) and its mycelia have long been valued in Asian cultures due to its therapeutic as well as culinary properties. The effect of different pH values, medium formulations on yield of dry mycelia and total amino acid content were studied. Submerged culture technique was employed to grow mycelia in Potato Dextrose Broth, PDB and PDB++ (PDB, yeast extract, malt extract, peptone, KH_2PO_4 and $MgSO_4.7H_2O$) media under different pH values. The amino acid composition of the mycelia was determined by High Performance Liquid Chromatography (HPLC) techniques. An optimum growth of the mycelia was observed on the PDB++ medium incubated at pH 6. The mycelia harvested had the highest content of amino acid in which the major component was glutamic acid.

Introduction

Lentinula edodes, commonly known as Shiitake mushroom, is a Basidiomycete fungus belonging to the order Agaricales and family Tricholomatacea. This mushroom is the second most popular edible mushroom worldwide and has delicious flavour, great nutritional value and immunity enhancing components (Jong and Birmingham 1993). This mushroom has been used for more than 2,000 years in Europe and Asia, where they were cultivated for consumption under primitive conditions (Flegg et al. 1985)

Mushrooms, like other microorganisms, have the potential to produce large amounts of protein depending on genera and species selected and the culture conditions used. Culture medium factors such as medium components and pH are known to influence microbial growth. Generally, mushroom has good tolerance to low water activity, pH and high osmotic pressure (Atlas 1997). Although the effect of pH on the growth of mycelia depends to some extent on the organism used, pH 4 – 7 seemed to give the best yields of mycelia (Michael et al. 2001).

Most researchers have spent their efforts on cultivating edible or medicinal mushrooms on solid artificial media (for fruit body production) rather than in submerged cultures (for mycelia production) (Bum et al. 2004). It usually takes several months to produce the fruiting body of *L. edodes* in solid cultures. Many attempts

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are being made to obtain useful cellular materials or to produce effective substances from cultured mycelia (Fan and Chun 1998). According to Friel and McLoughlin (2000), submerged cultures may have potential advantages of higher production of mycelia in a compact space and shorter time with lesser chances for contamination.

The objective of this study was to evaluate the effects of various pH on the growth of mycelial biomass and amino acid production in batch cultures.

*Plate 1. **Lentinula edodes** mycelia*

*Plate 2. Submerged cultures of **Lentinula edodes** mycelia*

Materials and methods

Stock culture development

The mushroom, *Lentinula edodes,* was purchased from Vita Agrotech Sdn. Bhd. Spores from the mushroom fruiting bodies were collected and inoculated on potato dextrose agar (PDA) and incubated at 25 °C for 14 days until mycelial growth was observed (*Plate 1*). The mycelia was isolated and the growth maintained as slope on PDA at 25 °C for one week (dark conditions) and then stored at 4 °C as stock culture. Mycelia from the stock culture was inoculated on fresh PDA and incubated at 25 °C for one week in the dark before use.

Growth media

A standard media, potato dextrose broth (PDB: 24 g) and a modified PDB (PDB++) were employed in this study. The modified medium PDB++ consisted of PDB: 20 g, yeast extract: 5 g, malt extract: 5 g, peptone: 5 g, potassium phosphate: 2 g and magnesium sulphate heptahydrate: 0.5 g (Aminuddin et al. 2003).

Inoculation procedure

Two weeks old mycelia agar disc (9 mm diameter) were 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. The pH of the broth was adjusted with 1 N HCl and 1 N NaOH to obtain different pH values (4, 6, 7 and 9) during the cultivation (*Plate 2*). Three replicates were performed for each pH value.

Determination of biomass

Mycelial growth 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 amount of distilled water. The mycelia were then freeze-dried for 5 – 8 h (Labconco 195, England) to a constant weight. The mycelial growth was measured every 3 days.

Amino acid analysis

Individual amino acids were determined after digestion of mycelia 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. A 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).

Derivatisation was carried out according to Cohen and De Antonis (1994) using borate buffer and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (ACCQ-tag reagent). Seventy microlitres of borate buffer and 10 µl of sample were added and mixed. 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 derivatisation process.

The amino acid content of mycelia was analysed either as 6 N HCl or performic acid hydrolysate and quantitatively determined using the Waters 510 HPLC system (Waters, USA) and detected by the Waters 470 fluorescence detector at E_λ and E_m of 250 nm and 395 nm respectively. Ten microlitres 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 litres 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 was as follows:

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

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

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

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 (g)
Rf = Response factor

Statistical analysis

The experiment was designed using completely randomised design (CRD). For data analyses, the SAS (Statistical Analysis System) program was used (SAS Inst. 1985). The values obtained were subjected to analysis of variance (ANOVA) and mean separation was done using Least Significant Difference (LSD) to determine differences among samples (Gomez and Gomez 1984).

Results and discussion

The pH of the culture is an important parameter that has been reported to affect fungal morphology (Gibbs et al. 2000). In natural environment, fungi can be exposed to a wide range of pH, with daily and seasonal variations, so active growth may not always be possible.

The mycelium was cultivated at various pH values (4, 6, 7 and 9) to determine the optimal pH for mycelial growth and amino acid production of *L. edodes*. Proper aeration was also assured through sterile cotton plug of the cultivation flask. The optimal pH for mycelial growth in PDB submerged culture was at pH 6 which produced 0.044 ± 0.004 g/biomass after 21 days of incubation

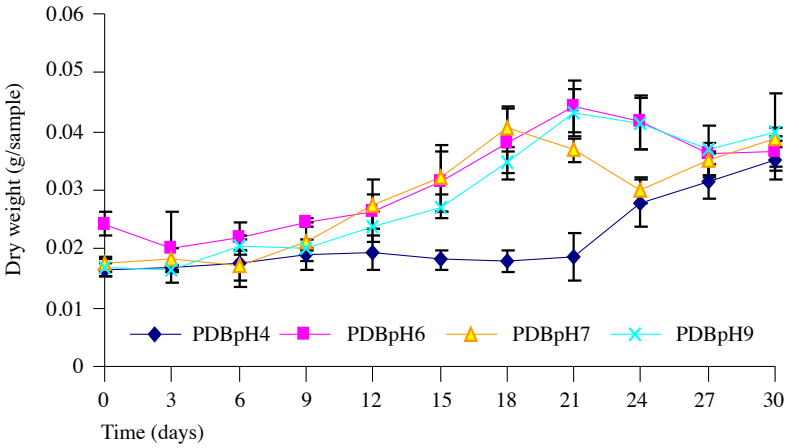

Figure 1. Effect of different pH on mycelial growth in PDB submerged cultures of ***Lentinula edodes*** *mycelia. The error bars ± in the figure indicate the standard deviations (SD) from triplicate samples*

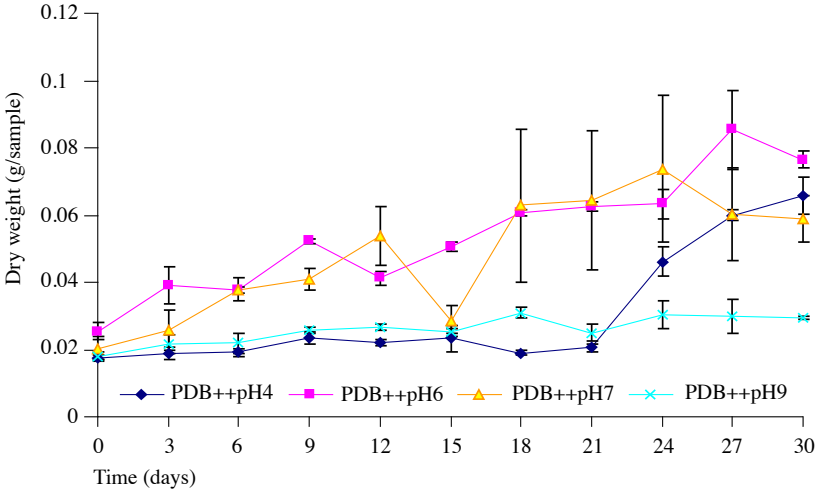

Figure 2. Effect of different pH on mycelial growth in PDB++ submerged cultures of ***Lentinula edodes*** *mycelia. The error bars ± in the figure indicate the standard deviations (SD) from triplicate samples*

(*Figure 1*). However, there was not much difference ($p > 0.05$) between yields of mycelia obtained at pH 9 and pH 7, which produced 0.043 ± 0.004 g/biomass at day 21 and 0.04 ± 0.003 g/biomass at day 18 compared to pH 6. The growth of mycelia in the PDB incubated at pH 4 was rather static at day 3 to 21 incubation, but slowly increased until the 30th day. Due to the low pH, the mycelial growth was slower compared to pH 6, 7 and 9.

The effect of different pH on mycelial growth in PDB++ is shown in *Figure 2*. The optimum growth of mycelium in PDB++ was observed when the suspension culture was incubated at pH 6 for 27 days producing 0.085 ± 0.012 g/biomass. This was followed by 0.074 ± 0.022 g/biomass

(24 d) and 0.062 ± 0.009 g/biomass (30 d) at pH 7 and 4 respectively. The results showed that there were no significant differences (p >0.05) between yield of mycelia obtained at pH 6 and 7. It was observed that when the pH was too low, the cell metabolism activity would reduce and inhibited the cell growth while at higher pH there was shorter growth period. The decrease in the growth rate may reflect either a disruption of metabolic regulations or death of cells. According to Michael et al. (2001), addition of a permeant weak acid to the medium will cause an acidification of the cytoplasm, as it will cross the plasma membrane as free acid and ionize to its anion and proton within the cell. This acidification could inhibit the cell.

The growth of mycelia in PDB medium was relatively lower than PDB++ medium. Most microorganisms require carbon and nitrogen sources or a mixture of amino acids to support the mycelial growth. 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 mycelia. But too high concentrations of glucose can have an inhibitory growth effect on several mushrooms. According to Goldberg and Williams (1991), the most favorable pH range for the growth of mushrooms is pH 6 – 7, similar to results obtained in this study. Thus, the growth medium and pH affect the rate of cell reactions, nutritional requirement, the nature of metabolism and also biomass composition.

The amounts of each amino acid produced by the mycelia are shown in *Tables 1* and *2*. The HPLC analysis showed that the submerged culture of *L. edodes* mycelia contained 16 amino acids namely aspartic acid, serine, glutamic acid, glycine, histidine, arginine, threonine, alanine, proline, tyrosine, valine, methionine, lysine, isoleucine, leucine and phenylalanine. The most abundant amino acid in both PDB and PDB++ cultures was glutamic acid. Mycelial biomass in PDB++ medium incubated at pH 6 contained the highest amount of amino acid (p >0.05) compared to other pH and media. It produced 20.79 g/100 g sample of amino acid during the 27 days of incubation, followed by PDB++ pH 7 (15.1 g/100 g sample), PDB pH 6 (11.57 g/100 g sample), PDB++ pH 4 (10.48 g/100 g sample) and PDB++ pH 9 (10.47 g/100 g sample). This result implies that the highest yield of the mycelium not only varies with different carbon and nitrogen source, but also with different pH. According to Goldberg and Williams (1991), 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 also due to the influence of the amino acid content on flavour.

Eight essential amino acids were found in mycelia cultured in both PDB and PDB++ media, namely, histidine, threonine, valine, methionine, lysine, isoleucine, leucine and phenylalanine. The essential amino acids in PDB mycelia biomass culture represented 2.4 to 4.52 g/100 g sample of the total amino acids, while mycelia biomass in PDB++ incubated at pH 6 contained 2.91 to 6.47 g/100 g sample. Results showed that mycelia in PDB++ incubated at pH 6 contained the highest amount of essential amino acids compared to other parameters. It also indicated that nitrogen and carbon sources uptake and assimilation by mycelia are influenced by environmental factors such as pH and temperature for synthesis of the essential and non-essential amino acids. The components of media, such as mineral salts and inorganic salts, influenced the flavour and amino acid composition of mycelia obtained especially glutamic acid. Based on the analysis of amino acids in mushroom fruiting bodies and mushroom mycelia, which showed an adequate level of all but the sulfur-containing amino acids, it seems that mushroom mycelia can be expected to be beneficial as a food supplement for human or animal consumption in the same way as other microbial protein sources (Goldberg and Williams 1991).

Table 1. The composition of amino acid mycelia with different culture pH in PDB submerged cultures

Amino Acid (g/100 g sample)	pH 4	pH 6	pH 7	pH 9
Aspartic acid	0.58 ± 0.14b	1.16 ± 0.03a	0.60 ± 0.04b	0.64 ± 0.10b
Serine	0.32 ± 0.04b	0.69 ± 0.02a	0.36 ± 0.01b	0.39 ± 0.06b
Glutamic acid	1.83 ± 0.29ab	2.02 ± 0.13a	1.16 ± 0.04c	1.60 ± 0.12b
Glycine	0.34 ± 0.04b	0.47 ± 0.04a	0.35 ± 0.03b	0.37 ± 0.03b
Histidine	0.16 ± 0.01b	0.26 ± 0.02a	0.11 ± 0.01b	0.22 ± 0.05a
Arginine	0.77 ± 0.05ab	0.88 ± 0.01a	0.54 ± 0.01c	0.66 ± 0.16bc
Threonine	0.31 ± 0.02b	0.58 ± 0.02a	0.32 ± 0.01b	0.36 ± 0.06b
Alanine	0.52 ± 0.06b	0.82 ± 0.01a	0.29 ± 0.08c	0.57 ± 0.14b
Proline	0.55 ± 0.01b	0.69 ± 0.02a	0.65 ± 0.02a	0.55 ± 0.08b
Tyrosine	0.13 ± 0.01c	0.32 ± 0.01a	0.15 ± 0.01c	0.20 ± 0.02b
Valine	0.34 ± 0.01c	0.62 ± 0.01a	0.40 ± 0.01b	0.44 ± 0.05b
Methionine	0.04 ± 0.01c	0.06 ± 0.01b	0.06 ± 0.01b	0.08 ± 0.01a
Lysine	0.43 ± 0.02b	0.90 ± 0.03a	0.38 ± 0.02b	0.47 ± 0.11b
Isoleucine	0.31 ± 0.01c	0.66 ± 0.04a	0.35 ± 0.01bc	0.42 ± 0.09b
Leucine	0.50 ± 0.02b	0.83 ± 0.01a	0.47 ± 0.01b	0.77 ± 0.22a
Phenylalanine	0.33 ± 0.01b	0.61 ± 0.01a	0.31 ± 0.01b	0.51 ± 0.11a

PDB = Potato Dextrose Broth; PDB++ = Enriched Potato Dextrose Broth
Mean values in the same row with same letters are not significantly different (p >0.05)
The error bars ± in the table indicate the standard deviations (SD) from triplicate samples

Table 2. The composition of amino acid mycelia with different culture pH in PDB++ submerged cultures

Amino Acid (g/100 g sample)	pH 4	pH 6	pH 7	pH 9
Aspartic acid	0.96 ± 0.14c	2.12 ± 0.12a	1.22 ± 0.01b	0.81 ± 0.03c
Serine	0.42 ± 0.08c	1.04 ± 0.05a	0.69 ± 0.02b	0.40 ± 0.01c
Glutamic acid	2.46 ± 0.19b	4.14 ± 0.42a	2.45 ± 0.02b	2.38 ± 0.18b
Glycine	0.80 ± 0.01c	1.57 ± 0.05a	1.69 ± 0.10a	1.37 ± 0.13b
Histidine	0.16 ± 0.03b	0.32 ± 0.04a	0.30 ± 0.06a	0.15 ± 0.04b
Arginine	0.82 ± 0.05c	1.47 ± 0.01a	1.27 ± 0.06b	0.45 ± 0.05d
Threonine	0.36 ± 0.01c	0.90 ± 0.02a	0.59 ± 0.02b	0.32 ± 0.04c
Alanine	0.82 ± 0.05b	1.37 ± 0.02a	1.28 ± 0.20a	1.02 ± 0.17b
Proline	1.05 ± 0.05c	2.10 ± 0.15a	1.58 ± 0.02b	0.97 ± 0.14c
Tyrosine	0.19 ± 0.01c	0.51 ± 0.04a	0.32 ± 0.01b	0.16 ± 0.01c
Valine	0.44 ± 0.02c	0.99 ± 0.07a	0.81 ± 0.01b	0.48 ± 0.04c
Methionine	0.07 ± 0.01d	0.21 ± 0.01a	0.15 ± 0.01b	0.09 ± 0.01c
Lysine	0.44 ± 0.07bc	0.97 ± 0.17a	0.63 ± 0.04b	0.32 ± 0.06c
Isoleucine	0.39 ± 0.05c	0.94 ± 0.09a	0.64 ± 0.02b	0.45 ± 0.06c
Leucine	0.65 ± 0.01c	1.20 ± 0.07a	0.91 ± 0.02b	0.65 ± 0.05c
Phenylalanine	0.45 ± 0.01c	0.94 ± 0.04a	0.57 ± 0.02b	0.45 ± 0.06c

PDB = Potato Dextrose Broth; PDB++ = Enriched Potato Dextrose Broth
Mean values in the same row with same letters are not significantly different (p >0.05)
The error bars ± in the table indicate the standard deviations (SD) from triplicate samples

Conclusion

The optimum pH for both mycelial growth and highest production of amino acid was at pH 6 grown in PDB++ medium. Mycelial growth and amino acid composition at pH 4 and 9 were more moderate when PDB or PDB++ medium was used. A total of 16 amino acids were identified in mycelia submerged culture. The most abundant amino acid in mycelial culture was glutamic acid. The study showed that the technique can be applied to produce high yield of mycelial biomass and thus increase the production of our local mushroom industry.

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Abstrak

Di negara Asia, *Lentinula edodes* (Shiitake) dan miselianya telah terkenal sejak berzaman dalam industri makanan dan perubatan. Kajian terhadap kesan pH yang berbeza, formulasi medium terhadap biojisim miselium kering dan kandungan jumlah asid amino telah dijalankan. Teknik kultur terendam telah digunakan bagi pertumbuhan miselia dalam kaldu dekstrosa kentang, PDB dan PDB++ (PDB, ekstrak yis, ekstrak malta, pepton, KH_2PO_4 dan $MgSO_4.7H_2O$) pada pH yang berbeza. Komposisi asid amino miselia ditentukan menerusi Kromatografi Cecair Prestasi Tinggi (HPLC). Medium PDB++ menghasilkan pertumbuhan miselia yang optimum pada pH 6. Miselium yang dituai pada media tersebut turut mengandungi komposisi asid amino yang tertinggi dengan asid glutamik merupakan komponen yang utama.

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