Research Article



# The effects of nutritional and fermentation conditions on mycelium growth of *Cordyceps militaris* in liquid culture

## Abstract

*Cordyceps militaris* is a valuable medicinal mushroom. In this study, nutritional compositions and fermentation conditions, were investigated to evaluate the growth of this mushroom in liquid culture. Among the investigated carbon sources, glucose showed highest efficiency with colony density reaches 16.8 colonies per ml, colony diameter reaches 1.4mm, and mycelial biomass reaches 12.42g/l. The most suitable source of nitrogen is silkworm pupae extract with the colony density reaches 19.2colonies/ml, colony diameter reaches 1.6mm, and mycelial biomass reaches 15.53g/l. The most suitable sources of mineral salts are $K_2HPO_4$ and $MgSO_4.7H_2O$. The pH of the culture medium was determined at 6. The appropriate ratio of seeding stock for biomass production was determined at 5.108/ml.

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## Introduction

Cordyceps are medicinal mushrooms belonging to the Ascomycetes group, which has long been used in Eastern countries such as China, Korea, and Vietnam.[1,2] Among approximately 600 Cordyceps species, *C. sinensis* and *C. militaris* are the most common. Although *C. sinensis* is preferred, is has been shown that *C. sinensis* and *C. militaris* contain quite similar biologically active ingredients.[3–5] *C. militaris* extracts has been shown containing adenosine, cordycepin, D-mannitol, and exopolysaccharides... These compounds possess pharmacological properties including anti-inflammatory properties,[6] anti-growth and anti-metastasis of cancer cells,[7,8] improving insulin secretion and anti-diabetes,[9] protecting the liver.[10] Recently, due to difficulties in exploiting harvesting natural Cordyceps, artificial culture considered as suitable alternative approaches to produce these medicinal mushrooms. Toward this goal, *C. militaris* has been cultivated and consumed as an alternative to natural *C. sinensis*.

The morphology of mycelium in submerged culture has been shown to play an important role in the fermentation process and the transformation of some bioactive substances.[11–13] During the fermentation process, some fungi can grow as free mycelium or aggregate into globular bacteria. The form of growth is determined by a number of factors such as nutrient composition, density of spores and physical effects during culture.[11,14,15] Cultivation conditions significantly influent the growth of mycelium, including the morphology of mycelium and metabolize the biologically active substances.[16,17] The nutritional composition of culture medium plays an important role in metabolism, providing energy for the cell's living activities. Carbon is a key component in submerged fermentation environment of *C. militaris* ensuring the growth and synthesis of necessary biological compounds. Nitrogen was shown to play a role in biosynthesis for enzymes in the biosynthesis of enzymes necessary for the primary and secondary metabolism of fungi.[18] However, there is limitted number of extensive research on the relationship between morphological parameters and productivity of fermented products.

In this study, different sources of carbon, nitrogen and mineral salts were investigated to find suitable substrate. The colony density introduced into culture and pH of culture medium were also investigated to find the most efficient ratio. Fermentation conditions are controlled at a temperature of 23°C, shaking rate is 150 rpm, using 500 ml flask as fermentation tank containing 250ml of nutrient medium.

## Material and methods

### Inoculum preparation

The isolate of *Cordyceps militaris* NBRC 9787 used in this study was purchased from the Japanese NITE Biological Resource Center (NBRC). The stock culture was activated on PDA potato agar medium (200g potato, agar 20g/L, glucose 20g/L) and stored at 23°C for 7 days. Subsequently, 10ml of sterile distilled water is added to the inclined agar tube and filtered through sterile absorbent cotton. The suspension containing spores is used for evaluation of different culture conditions.

### Effect of fermentation conditions on the growth of mycelium

Multiple factors including nutritional compositions (carbon, nitrogen, and mineral salts) and fermentation conditions (pH and seed ratio) were taken into account to evaluate the effectiveness of liquid propagation. The fermentation process was carried out in a 500ml flask containing 250ml of medium, controlled at 23°C for 7 days, shaking rate of 150rpm. Each experimental element is described in detail as follows.

### Carbon sources

Nutrition affects throughout the growth process of mycelia. It is an important determinant of seed growth and quality. Five different types of sugar were investigated including fructose, sucrose, maltose, lactose, and glucose.

### Nitrogen source

Nitrogen is the essential source of nutrition in *C. militaris* breeding. It plays a role in the biosynthesis of enzymes necessary for primary and secondary metabolism of fungi. The investigated nitrogen sources including peptone, high yeast, silkworm pupae extract, sodium nitrate, and ammonium nitrate.

### Mineral elements

Mineral elements also play an essential role in the growth of mycelium in liquid medium. Mineral elements related to catalytic functions to synthesize several enzymes for the growth of mycelium. The mineral elements surveyed in this study including $KH_2PO_4$, $K_2HPO_4$, $Ca(NO_3)_2$, $CaCl_2$, KCl, $MgSO_4$, and $FeSO_4$.

### Concentration of seed culture

Suspension containing spores was added to the culture medium at $10^8$, $3x10^8$, $5x10^8$ and $7x10^8$ ans used as seed culture. Number of spores was determined by red cell counting method using Hematocytometer chamber.

### Initial pH

pH has effect on cell functions, nutrient adsorption, morphology and cell structure, salt solubility and ionic state of substrate, enzyme activity and biosynthesis. In this study, initial pH was investigated at 5, 5.5, 6, 6.5, 7, 7.5. The pH of the medium is adjusted using 1N HCN or 1N NaOH.

### Statistical analysis

To evaluate the effectiveness of fermentation process, a number of factors were investigated including colony density (colonies/ml), colony diameter (mm) and mycelium biomass (g/L). Each experiment was repeated 3 times. Abnormal values were removed by Duncan method. Statistical analysis, ANOVA performed by IRISTART 4.0 and Microsoft Excel 2013 software.

## Results and discussion

### Effects of nutrient sources

The effect of carbon sources on the growth of *C. militaris* mycelium in liquid culture was presented in Table 1. Five different types of sugars including fructose, sucrose, maltose, lactose, and glucose were tested. The results indicated that there is no difference in colony diameter, however the density and biomass of the mycelium differed between carbon sources. The best growth rate was observed with the medium contains glucose, followed by sucrose and the lowest is lactose. Previous reports indicated that carbon is an important source of energy for *C. militaris* cells. This study observed the similar results with those of Mao et al.[19] Sucrose, composed of 2 molecules α-D-glucopyranozyl and β-D-fructofuranozit, yielded similar fermentation efficiency with glucose. Lactose, composed of 1 β-glucose molecule and 1 β-galactose molecule, yielded the lowest growth efficiency. Decrease of growth of mycelia leads to reduced mycelium biomass.[19] Therefore, the nature of each different carbon source will affect the growth of the mycelium.

Five nitrogen sources were added to the liquid culture medium. They were divided into 3 groups including artificial synthetic nitrogen source (pepton and yeast extract), natural nitrogen source (silkworm pupae extract), and inorganic nitrogen source (sodium nitrate and ammonium nitrate) (Table 1). The strongest and the worst growth rate were observed with natural and inorganic nitrogen group, respectively. Artificial synthetic nitrogen group showed similar efficiency as natural nitrogen group. Therefore, in term of convenience, pepton and yeast extract were recommended for industrial scale. Importantly, our data indicated that natural nitrogen source, silkworm pupae extract, was the best suitable carbon source for the growth of *C. militaris*. This result is somewhat in agreement with a previous report that showed another kind of natural nitrogen source, the corn extract, was the best.[20]

Mineral salts have been shown to be important for the growth of *C. militaris*.[15,21] In this study, different types of salts were investigated at concentration of 1g/L to evaluate the effects on growth of the mycelium in liquid culture. Among the tested minerals, $K_2HPO_4$ and $MgSO_4{\cdot}7H_2O$ yielded similar results and achieved the best fermentation efficiency (Table 1).

### Effect of concentration of spores of seed culture

The concentration of spores in seed culture affects the size and density of mycelia pellets and the ability to synthesize cordycepin. In the present study, the concentration of spore was tested at $10^8$, $3x10^8$, $5x10^8$ and $7x10^8$. As shown in Figure 1, seed density affect the growth characteristics of mycelia. The initial amount of inoculum is $10^8$ spores/ml leads to the low efficiency, resulted as large colony diameter but low density and biomass. Increasing the rate of inoculated seeds resulted in higher density of colonies, however the diameter of the colonies tended to be decreased (Figure 1B). The cause of this phenomenon is due to high cell density leading to competition of nutrition and hindering the barrier of transport, reducing air circulation, reducing contact between mycelium and nutrient molecules in environment. The biomass of the mycelium reached the highest level at the threshold of $5x10^8$ spores/ml. The optimal threshold has previously been determined at $3x10^8$ spores/ml.[15] The difference may be due to the growth characteristics of two different *C. militaris* strains.

**Table 1** Effects of nutrient sources on the growth of *C. militaris*

| | Colony density (colonies/ml) | Colony diameter (mm) | Biomass of mycelium (g/L) |
|---|---|---|---|
| Cacbon source | | | |
| Fructose | 9.4 | 1.3 | 11.92 |
| Glucose | *16.8* | *1.4* | *12.42* |
| Maltose | 11.1 | 1.4 | 10.73 |
| Sucrose | 14.4 | 1.2 | 12.10 |
| Lactose | 7.0 | 1.5 | 10.03 |
| Nitrogen source | | | |
| Peptone | 17.3 | 1.4 | 13.34 |
| Yeast extract | 17.9 | 1.4 | 13.38 |
| Silkworm pupae extract | *19.2* | *1.6* | *15.53* |
| Natri nitrat | 10.4 | 0.9 | 11.16 |
| Amoni nitrat | 11.5 | 0.8 | 11.46 |
| Mineral | | | |

Table Continued

| | Colony density (colonies/ml) | Colony diameter (mm) | Biomass of mycelium (g/L) |
|---|---|---|---|
| $K_2HPO_4$ | 15.6 | 1.5 | 16.4 |
| $Ca(NO_3)_2$ | 11.2 | 1.1 | 11.5 |
| $CaCl_2$ | 13.1 | 1.2 | 12.6 |
| KCl | 10.5 | 0.9 | 11.2 |
| $MgSO_4.7H_2O$ | 15.1 | 1.4 | 15.5 |
| $FeSO_4$ | 8.6 | 0.7 | 10.3 |

The concentration of spores in seed culture affects the size and density of mycelia pellets and the ability to synthesize cordycepin. In the present study, the concentration of spore was tested at $10^8$, $3x10^8$, $5x10^8$ and $7x10^8$. As shown in Figure 1, seed density affect the growth characteristics of mycelia. The initial amount of inoculum is $10^8$spores/ml leads to the low efficiency, resulted as large colony diameter but low density and biomass. Increasing the rate of inoculated seeds resulted in higher density of colonies, however the diameter of the colonies tended to be decreased (Figure 1B).

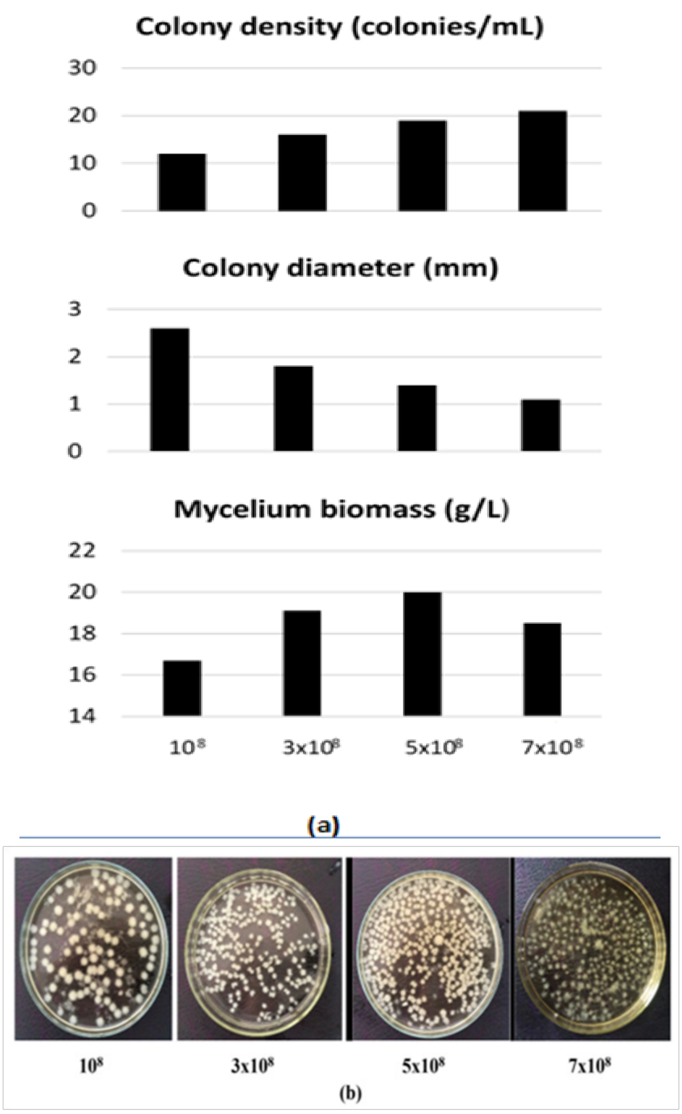


**Figure 1** Effect of concentration of spores of seed culturen on the growth of *C. militaris* mycelium.

The cause of this phenomenon is due to high cell density leading to competition of nutrition and hindering the barrier of transport, reducing air circulation, reducing contact between mycelium and nutrient molecules in environment. The biomass of the mycelium reached the highest level at the threshold of $5x10^8$spores/ml. The optimal threshold has previously been determined at $3x10^8$ spores/ml.[15] The difference may be due to the growth characteristics of two different *C. militaris* strains.

The initial pH has an effect on fungal cell membrane functions including the uptake of nutrients and other products of biosynthesis. According to previous studies, the optimal initial pH values for the growth of *C. militaris* vary in the range of 4 to 7. The pH change between different isolates of *C. militaris* is explained by the difference in growth rate, metabolism and nutritional needs for each specific isolate. In this study, we observed the optimal growth of *C. militaris* NBRC 9787 at pH 6, with colony diameter reached 1.6mm, colony density reached 25.3colonies/ml, and mycelium biomass reached 17.3g/L (Figure 2).

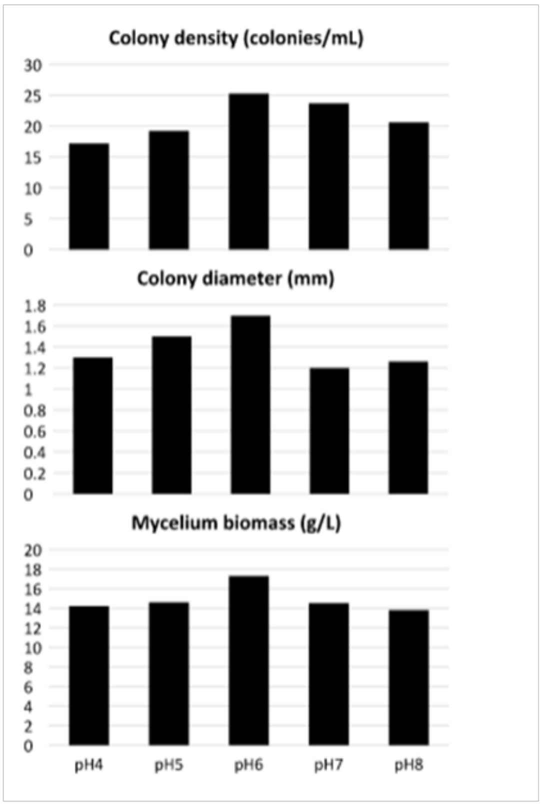


**Figure 2** Effect of pH on the growth of *C. militaris* mycelium in liquid culture.

## Conclusion

In the present study, the effects of nutritional compositions to the growth of *C. militaris* mycelia in liquid culture were investigated. We found that the most suitable carbon source is glucose with density of colonies reached 16.8colonies/ml, colony diameter reached 1.4mm, and mycelium biomass reached 12.42g/L. The most suitable source of nitrogen is silkworm pupae extract with the colony density reached 19.2colonies/ml, the diameter of the colony reached 1.6mm, and the mycelium biomass reached 15.53g/L. The most suitable sources of mineral salts are $K_2HPO_4$ and $MgSO_4.7H_2O$. Investigation of the fermentation conditions showed that the suitable seeding ratio is

$5x10^8$spores/ml. This ratio yielded the best fermentation effect with the colony density reached 19colonies/ml, the diameter of the colony reached 1.4 mm, and mycelium biomass reaches 20g/L. Finally, pH 6 is the suitable threshold for the growth of *C. militaris*.

## Acknowledgments

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## Conflicts of interest

The author declares there are no conflicts of interest.

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