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BIOMASS PRODUCTION OF FUNGUS *Termitomyces clypeatus* IN A STIRRED-TANK BIOREACTOR

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ABSTRACT

Termitomyces clypeatus is a fungus species, which has been used as food and medicinal mushroom. In the current study, submersed fermentation of the fungus in a stirred-tank bioreactor was determined for its culture biomass and nutrient contents. *T. clypeatus* obtained its highest biomass after from 13 to 15 days of cultivation, when the dry biomass of its mycelium system was more than 6%. Agitation speeds of 150 or 180 rpm were the most suitable for the fungus. The determination of chemical compositions showed that the dry biomass of its mycelium system had high protein content, with 57.8% on its dry-weight basis. Besides, 11 types of amino acids higher than 0.1% of wet weight culture were found in fermentation products. Moreover, dried mycelia of the fungus contained 27.4% carbohydrates. The protein and carbohydrate containing in the mycelium and fruit body of *T. clypeatus* were not statistically different. These results showed that a stirred-tank bioreactor could be applied to culture the fungus.

Keywords: Amino acids, chemical compositions, stirred-tank bioreactor, submersed culture, *T. clypeatus*.

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INTRODUCTION

Termitomyces clypeatus, a fungus species of basidiomycete fungi, has a local name "Nam Moi". This mushroom is a valuable food due to its deliciousness and nutritional contents. The fungus is not only consumed in Vietnam but also in other countries (Karun & Sridhar, 2013; Dutta & Acharya, 2014). A previous study presented that *T. clypeatus*'s fruit body contained protein, carbohydrates, fat, ascorbic acid and other components (Ogundana & Fagade, 1982). Besides, the fungus produces industrial enzymes with promising thermal stabilities (Ghorai et al., 2009). *T. clypeatus* has also been known as a medicinal mushroom because of its antibacterial effects (Giri et al., 2012), anti-proliferative and antibacterial potential (Majumder et al., 2014), supportive treatment of chickenpox (Acharya et al., 2014), high antioxidant activities (Mau et al., 2004; Pattanayak et al., 2015). Moreover, the fungus produces serine protease (AkP), an anticancer enzyme (Majumder et al., 2016).

The application of the liquid culture method to produce edible and medicinal fungi has been successfully achieved with varieties of fungi. The results of the laboratory experiments showed that the majority of mycelium systems grew well in suitable liquid media and environmental conditions (Hasan et al., 2012). Submerged fermentation of fungi which are rare and difficult cultivable is a suitable method to produce mushroom biomass with reasonable quality (Kwon et al., 2009).

Termitomyces clypeatus cannot currently be grown by traditional methods, so the submerged fermentation is an effective alternative to culture and yield its biomass (Lu et al., 2008). However, the production of *T. clypeatus* cultured in liquid media at laboratory and industrial scales has not been conducted. Moreover, submerged fermentation techniques are typically preferred due to their easy application and better batch-to-batch control. The liquid fermentation of macrofungi has been conducted in Vietnam, such as fungi *Clitocybe maxima* (Ngo Xuan Nghien & Nguyen Thi Bich Thuy, 2016), *Pleurotus eryngii* and *Trametes versicolor* (Nguyen Thi Bich Thuy, 2014). However, liquid fermentation for producing *T. clypeatus* has not been published yet. Therefore, this study aims to evaluate the production of fungus biomass by using a stirred-tank bioreactor. The effects of media components and shaken conditions on the growth and nutrient values of *T. clypeatus* were also determined.

MATERIALS AND METHODS

Culture media and fungus

Two liquid media (MT1 and MT2) were prepared to culture *T. clypeatus*. The components of MT1 were 5.59% glucose, 0.48% pepton and 0.16% KH_2PO_4. The nutrient values of MT2 were 6.99% glucose, 0.60% pepton and 0.2% KH_2PO_4. MT2 was considered as the optimum for *T. clypeatus* (Nguyen Thi Ngoc Nhi & Tran Nhan Dung, 2018). The nutrient values of MT1 were prepared at 80% of MT2. The pH was adjusted at 5.0 using H_2SO_4 and NaOH. All chemicals with high purity were purchased from India and China.

The fungus *T. clypeatus* (KU569480.1) prepared according to previous studies (Nguyen Thi Ngoc Nhi & Tran Nhan Dung, 2016, 2018) was used in all experiments.

Stirred-tank bioreactor

A stirred-tank bioreactor (Bon Mua Biotech Company, Vietnam) with a volume of 60 L was used to culture *T. clypeatus*. The 60 L bioreactor was connected to an air compressor, a boiler and a chiller by pipes (Fig. 1). Fluid and air flowed through the pipes were also controlled by valves. The agitation system consisted of a stirring motor with impeller blades. The pH was automatically controlled using H_2SO_4 and NaOH at an acid/base site. CIP (Clean-in-place) was the equipment used for automated cleaning the interior surfaces of bioreactor, pipes and vessels. A sensor was the equipment used for monitoring bioprocesses. The bioreactor was manually controlled at a control board.

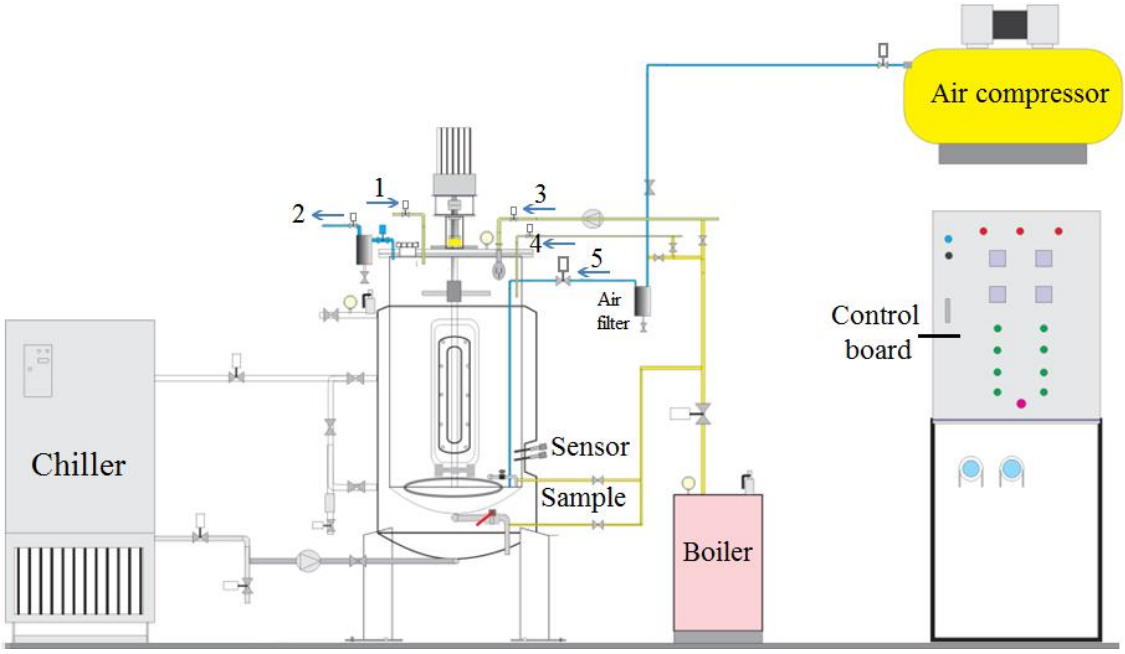

Figure 1. Scheme of the stirred-tank bioreactor. (1) Air outlet, (2) Feed, (3) CIP, (4) Acid/base and (5) Air inlet

Fermentation process

Liquid media (35 L), MT1 or MT2, were added into the reactor. The media were autoclaved at 121 ºC for 30 min by the boiler, and then cooled by the chiller. *T. clypeatus* was inoculated at 5%. The fermentation process was conducted at 28 ºC. Air was introduced at the bottom of the reactor at 0.4 vessel volume of per minute (vvm). The stirring rate was controlled at 120, 150 and 180 revolutions per minute (rpm). During the fermentation, the pH was controlled at 5.0. The experiment was conducted with three replicates.

Samples were collected at the sample site at the bottom of the bioreactor every day to determine the yield of dried biomass and concentrations of carbohydrates, protein, fat, vitamin C and all amino acids. Each time, 50 mL of solution was collected for measuring chemical compositions.

The solution was passed through the filter-paper. Mycelia which were not moved through filter-paper were collected, dried by a dry cooling machine (BMB-MSL150, Bon Mua Biotech Company, Vietnam) at 50 ºC until constant mass to obtain dried biomass of mycelia.

***T. clypeatus*'s fruit body collection**

Fruit bodies of fungus *T. clypeatus* were ramdomly collected from Binh Duong Province described in the previous report (Nguyen Thi Ngoc Nhi & Tran Nhan Dung, 2016). The fungus was transformed into a laboratory for chemical analysis. Fruit bodies were cleaned and then died until constant weight before chemical analysis.

Analytical methods

Chemical contents of mycelia and fruit bodies of the fungus were determined. The methods to determine all components are shown in Table 1. The analyzed results were shown on wet and dry-weight basis.

Statistical analysis

Data are shown as the mean ± standard deviation. The Minitab program version 16 was used to analyze variance, and significant differences ($p < 0.05$) were calculated using Tukey's test.

Table 1. Methods to determine chemical compositions of the fungus

Components	Methods
Carbohydrates	AOAC 986.25 mod
Protein and fat	FAO (1986)
Vitamin C (Acid ascorbic)	AOAC 2012.21 mod
Water	Internal method (EHC-TP2-048)
Alanin, acid aspartic, cystine, acid glutamic, glycin, histidin, 4-hydroxyprolin, isoleucin, leucin, lysin, methionine, phenylalanin, prolin, serin, threonin, tyrosin, valin and total of amino acid	Internal method (EHC-TP1-044) (GC-FID)
Tryptophan	Internal method (EHC-TP1-146) (GC-FID)
Arginine	AOAC 994.12 mod

RESULTS AND DISCUSSION

Effects of nutrient values on biomass of *T. clypeatus* cultured in the stirred-tank bioreactor

The growth of fungi at various nutrient components is usually different. This experiment determined the effects of nutrient contents on biomass production at 150 rpm. The biomass of *T. clypeatus* cultured in MT1 and MT2 with different nutrient concentrations was compared (Table 2). The biomass values of the fungus growing in both media were not statistically changed from 3rd to 5th day, but started to increase at the day of 6, and obtained the maximal yields on from 13th to 15th day. However, the biomass values were reduced at the following time. These results showed that *T. clypeatus* required 5 days of lag phase when it was being adapted to new media. The log phase was from 6th to 13th day. The death phase began on 16th day when culture media turning to dark color due to the death of fungus were observed.

The biomass values of fungus *T. clypeatus* cultured in MT2 were significantly higher than those in MT1 on 13th–15th day, even though the values were not statistically different at other collection times (Table 2). The use of an alternative medium (MT1) for economic purposes reduced mycelium production. These results indicated that higher nutrient contents in MT2 supported the growth of the fungus.

In a previous study, Elisashvili (2012) reported that a liquid culture of some medicinal mushrooms, such as *T. versicolor*, *Ganoderma lucidum* and *Lentinus edodes*, obtained the yields of 9.1, 10.5 and 5.6 g/L of mycelia in media containing glucose, respectively. Submerged culture of other several medicinal mushrooms obtained biomass from 0.4 to 9.6 g/L after 10−15 days (Kim et al., 2002). In another report, the dry biomass of *Termitomyces eurrhizus* was 21.1 g/L after 5 days (Zhang et al., 2002), which was significantly higher than that of *T. clypeatus* in our study.

From the data shown in Table 2, the changes of biomass were presented in figure 2. The regression equations of *T. clypeatus* cultured in MT1 and MT2 were $4.034 - 1.911x + 0.2745x^2 - 0.009351x^3$ and $4.483 - 2.207x + 0.3285x^2 - 0.01149x^3$, respectively. Both curves had high R-Sq values, indicating that the growth of fungus fitted well with realistic conditions. The biomass can be predicted based on the equations.

Table 2. The biomass of fungus *T. clypeatus* cultured in MT1 and MT2 media in the stirred-tank bioreactor. The experiment was conducted at an agitation speed of 150 rpm

Fermentation time (days)	Dry biomass (g/L)	
	MT1	MT2
3	$0.321^{p} \pm 0.028$	$0.306^{p} \pm 0.019$
4	$0.333^{p} \pm 0.028$	$0.329^{p} \pm 0.018$
5	$0.388^{p} \pm 0.024$	$0.479^{op} \pm 0.021$
6	$0.547^{op} \pm 0.033$	$0.749^{op} \pm 0.024$
7	$0.840^{nop} \pm 0.050$	$1.027^{no} \pm 0.091$
8	$1.380^{nm} \pm 0.034$	$1.797^{lm} \pm 0.159$
9	$1.994^{kl} \pm 0.191$	$2.503^{jk} \pm 0.195$
10	$2.899^{j} \pm 0.154$	$3.576^{i} \pm 0.100$
11	$3.810^{hi} \pm 0.268$	$4.674^{efg} \pm 0.154$
12	$4.758^{efg} \pm 0.276$	$5.860^{bc} \pm 0.158$
13	$5.271^{cde} \pm 0.330$	$6.378^{ab} \pm 0.156$
14	$5.469^{cd} \pm 0.360$	$6.591^{a} \pm 0.078$
15	$5.536^{c} \pm 0.168$	$6.549^{a} \pm 0.107$
16	$5.265^{cde} \pm 0.232$	$5.768^{c} \pm 0.314$
17	$4.788^{efg} \pm 0.412$	$4.892^{def} \pm 0.274$
18	$4.218^{gh} \pm 0.177$	$4.644^{fg} \pm 0.199$

Note: Different small superscript letters indicate statistically significant differences ($p < 0.05$). Data are means of the results from three individual experiments, and mean values and standard deviations are shown.

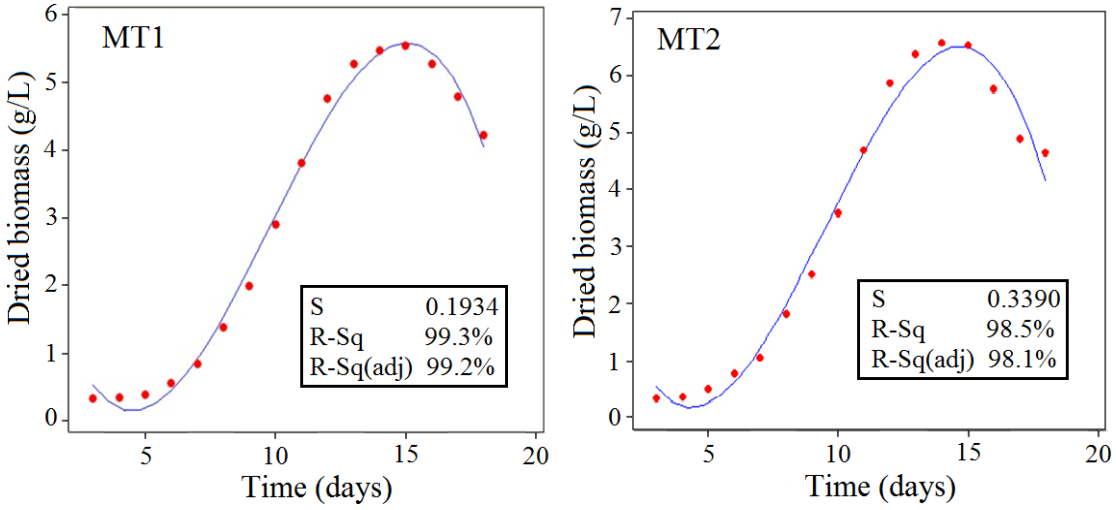

Figure 2. Died biomass of fungus *T. clypeatus* cultured in MT1 and MT2 media

Effects of agitation speeds on yielded biomass of fungus *T. Clypeatus*

To determine an optimal condition for fermentation, the effects of agitation speeds on yielded biomass of fungus were carried out. Table 3 shows that yielded biomass of the fungus was not statistically different at 120 rpm and 180 rpm before 6 days. However, the biomass productions at 150 rpm were not statistically different compared with those at 180 rpm at most fermentation times. The biomass values were highest after from 13th to 15th day, and no statistical

difference was observed at these times. In these times, dry biomass yields were more than 6%. However, the differences were found at the following time, when fungus biomass yields were significantly higher at 180 rpm. Therefore, fungus *T. clypeatus* should be cultured in MT2 medium, at 150 or 180 rpm for 13 days.

Table 3. Mycelium biomass of fungus *T. clypeatus* cultured in MT2 medium, in the stirred-tank bioreactor at 120 rpm and 180 rpm

Time (days)	Dry biomass (g/L)	
	120 rpm	180 rpm
3	$0.327^{r} \pm 0.013$	$0.353^{r} \pm 0.022$
4	$0.352^{r} \pm 0.021$	$0.354^{r} \pm 0.011$
5	$0.458^{r} \pm 0.021$	$0.506^{qr} \pm 0.029$
6	$0.736^{pq} \pm 0.033$	$0.782^{op} \pm 0.013$
7	$0.890^{oq} \pm 0.013$	$1.269^{mn} \pm 0.020$
8	$1.500^{m} \pm 0.055$	$1.946^{l} \pm 0.020$
9	$2.249^{k} \pm 0.047$	$2.551^{j} \pm 0.076$
10	$3.319^{i} \pm 0.041$	$3.690^{h} \pm 0.017$
11	$4.279^{g} \pm 0.041$	$4.911^{f} \pm 0.103$
12	$5.332^{e} \pm 0.021$	$5.911^{d} \pm 0.073$
13	$6.163^{c} \pm 0.054$	$6.633^{a} \pm 0.041$
14	$6.292^{c} \pm 0.050$	$6.695^{a} \pm 0.015$
15	$6.366^{bc} \pm 0.022$	$6.709^{a} \pm 0.015$

Note: Different small superscript letters indicate statistically significant differences ($p < 0.05$). Data are means of the results from three individual experiments, and mean values and standard deviations are shown.

The effects of agitation speeds on some species of genus *Termitomyces* were reported in several previous studies. *T. eurrhizus* was cultured at 120 rpm (Zhang et al., 2002), while *Termitomyces albuminosus* (Hu et al., 2001) and *T. clypeatus* (Ramrakhiani et al., 2011) were cultured at 150 rpm. For other fungal genera, *Cordyceps militaris* was suitable to culture in a bioreactor at 150−200 rpm (Wang et al., 2019), while *Ganoderma pfeifferi* should be cultutered at 120 rpm (Supramani et al., 2019).

Chemical components of *T. clypeatus*'s mycelia cultured in the stirred-tank bioreactor

The determination of chemical components is required to evaluate whether *T. clypeatus* can be used for food and medicine. Carbohydrate content was 26.08% of dry biomass. The fungus had high protein content, with around 3.749% (Table 4), equivalent to 54.92% on its dry-weight basis. However, fat, vitamin C, cystine/cysteine, 4-hydroxyprolin, methionine and tryptophan were not found in the fungus biomass.

The nutrient contents of other species of *Termitomyces* in previous studies were so different from our results in this study. For example, the components of *T. albuminosus* cultured in a submerged system were 49.18% protein, 10.75% carbohydrates and 8.47% fat of died mycelium biomass (Zhao et al., 1997).

Amino acids of *T. clypeatus* cultured in the bioreactor were quite rich, with 11 crucial types higher than 0.1% of wet weight. The total amino acids of mycelia were 2.160 ± 0.001%, equivalent to 31.6% of dry biomass. Several amino acids, i.e., histidin, isoleucin, phenylalanin and tyrosin, were negligible (Table 4). Amino acid contents of mycelia and fruit bodies of the species *Termitomyces* have been analyzed in previous publications. Total of amino acids of *T. albuminosus*'s mycelia

was 50.37 ± 0.26 mg/g (about 5.0% of dry weight) (Tsai et al., 2006), which was much lower than the result of this study. Amino acid contents of *T. eurrhizus* and *T. microcarpus* were 23.6% and 59.0% of their dried fruit bodies, respectively (Nakalembe & Kabasa, 2013). High protein as well as amino acids contents in this study indicated that cultured media and fermentation process were suitable for *T. clypeatus*.

Table 4. Chemical components of fungus *T. clypeatus* in its wet biomass of mycelia

Chemical components	Statistics (%)
Carbohydrates	1.780 ± 0.005
Fat	Not detected
Protein	3.749 ± 0.008
Vitamin C	Not detected
Moisture	93.91 ± 0.032
Total of amino acid	2.160 ± 0.001
Alanin	0.214 ± 0.004
Acid aspartic	0.173 ± 0.007
Cystine/Cysteine	Not detected
Acid glutamic	0.512 ± 0.008
Glycin	0.130 ± 0.006
Histidin	< 0.1
4-Hydroxyprolin	Not detected
Isoleucin	< 0.1
Leucin	0.130 ± 0.003
Lysin	0.130 ± 0.002
Methionine	Not detected
Phenylalanin	< 0.1
Prolin	0.111 ± 0.003
Serin	0.212 ± 0.002
Threonin	0.160 ± 0.001
Tyrosin	< 0.1
Valin	0.110 ± 0.002
Tryptophan	Not detected
Arginine	0.151 ± 0.002

Fruit body's nutrient values of fungus *T. Clypeatus*

Nutrient values of fruit bodies were analyzed and shown in Table 5. Carbohydrates, fat and protein were about 26.71%, 2.854% and 53% of dry biomass, respectively. Carbohydrates and protein in both mycelia collected from the reactor and fruit bodies of the fungus collected from the natural environment were not statistically different. Fat was not found in mycelia shown in Table 4 probably because of low concentration; however, the fat value was found in fruit bodies of the fungus.

The results of this study were so different from a study by Ogundana and Fagade (1982) who showed that dried fruit body of *T. clypeatus* had 31.4% protein, 32.4% carbohydrates, 4.96% fat and 14.3% acid ascorbic. The results of this study were so different from a study by Ogundana and Fagade (1982) who showed that dried fruit body of *T. clypeatus* had 31.4% protein, 32.4% carbohydrates, 4.96% fat and 14.3% acid ascorbic. The lower protein and higher carbohydrates, fat contents and acid ascorbic in the study of Ogundana and Fagade (1982) compared to our results probably because *T. clypeatus* was collected from different places and times.

For nutrient analysis of other species of *Termitomyces*, Ugbogu et al. (2018) showed that the fruit body of *Termitomyces robustus* had 18.44 ± 0.09% crude protein, 62.63 ± 0.53% carbohydrates, 2.88 ± 0.04% crude fat and 3.10 ± 0.15% acid ascorbic. Total fruit body protein and carbohydrate of *Termitomyces heimii* were 60.53 ± 0.01% and 22.74 ± 0.01% of dry weight respectively, while moisture was 81.1 ± 0.02%, and no data for fat content was showed (Ao & Deb, 2019). These results showed that the nutrients of species of *Termitomyces* were so different. The nutrient compositions of fungi depended on species, harvested time and natural conditions (Wani et al., 2010).

Table 5. Fruit body components of fungus *T. clypeatus*

Components	Statistics (%)
Carbohydrates	26.71 ± 0.015
Fat	2.854 ± 0.041
Protein	53.00 ± 0.150
Moisture	10.78 ± 0.027

CONCLUSION

The fungus *T. clypeatus* cultured in MT2 medium with agitation speeds of 150 or 180 rpm was obtained more than 6% of dry biomass on 13th day. Mycelia of *T. clypeatus* contained high amount of protein (57.8% on dry-weight basis), and had rich amino acids with 11 types higher than 0.1% of wet weight culture. Values of protein and carbohydrates of mycelia obtained from the stirred-tank bioreactor and fruit bodies collected from its natural habitats were not statistically different. This current study showed that the stirred-tank bioreactor can be applied to quickly culture *T.clypeatus*.

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