Submerged Cultivation of Mycelium with High Ergothioneine Content from the Culinary-Medicinal Golden Oyster Mushroom, *Pleurotus citrinopileatus* (Higher Basidiomycetes)

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ABSTRACT: The optimization of submerged culture of the culinary-medicinal golden oyster mushroom, *Pleurotus citrinopileatus*, was studied using a one-factor-at-a-time, two-stage stimulation and central composite rotatable design to produce mycelia with high ergothioneine content. The optimal culture conditions for mycelia harvested at day 22 were a temperature of 25 °C, an inoculation ratio of 5%, 2% glucose, 0.5% yeast extract, and adjustment of the initial pH value to 10. The biomass and ergothioneine content were 8.28 g/L and 10.65 mg/g dry weight (dw), respectively. The addition of an amino acid precursor increased the ergothioneine content of mycelia; cysteine was the most effective. In addition, the results obtained from central composite rotatable design showed that the recommended combination for cysteine, histidine, and methionine was 8, 4, and 0.5 mmol/L, respectively. The predicted ergothioneine content was 13.90 mg/g dw, whereas the experimental maximal ergothioneine content was 14.57 mg/g dw. With the addition of complex precursors and under optimal culture conditions, mycelia harvested at days 16–20 had higher ergothioneine content. Accordingly, the information obtained could be used to produce mycelia with high ergothioneine content.

KEY WORDS: medicinal mushrooms, ergothioneine, *Pleurotus citrinopileatus*, mushroom mycelia, response surface methodology

ABBREVIATIONS: CCRD, central composite rotatable design; dw, dry weight.

I. INTRODUCTION

Mushrooms are of high nutritional value and contain many bioactive compounds, including amino acids, dietary fiber, ergosterol, minerals, polysaccharides, phenolic components, and vitamins.1–3 One bioactive compound, ergothioneine (2-mercaptohistidine trimethylbetaine), is a water-soluble, naturally occurring amino acid that can form in some bacteria and nonyeast fungi but not in animals.4 The metabolic pathway to produce ergothioneine starts with the methylation of histidine by methionine to produce hercynine (histidine betaine); the sulfur atom is then incorporated from cysteine.5 In humans, the best known dietary sources of ergothioneine are mushrooms (0.1–1 mg/g) and meat.6,7 Ergothioneine has currently attracted awareness because of its identification as the biogenic key substrate of the organic cation transporter OCTN1 (*SLC22A4* gene).8 OCTN1 seems to have a pivotal protective role in monocytes and has been associated as a susceptible factor in the etiopathology of autoimmune disorders such as rheumatoid arthritis.9,10

*Pleurotus citrinopileatus* Singer (Pleurotaceae, higher Basidiomycetes) is a golden oyster mushroom, also known as *yu-huang-mo* in Chinese and
In general, the production of *P. citrinopileatus* includes a long period of cultivation in a plastic bag to grow fruiting bodies and a short period of submerged fermentation for mycelia and fermentation filtrate. The nutritional value and taste components of *P. citrinopileatus* in the forms of fruiting bodies and mycelia have been thoroughly studied. Many reports indicate that this mushroom possesses biological and pharmacological activities, such as antitumor activity, antigenotoxicity ability, antihyperglycemic effects, fatigue resistance, immunoenhancing ability, the ability to delay aging, and antihyperlipidemic and antioxidant effects. Chen et al. researched the ergothioneine content of edible and medicinal mushrooms, including 20 species of fruiting bodies and 20 species of mycelia. Their results showed that ergothioneine was detected in all samples, and *P. citrinopileatus*, *Pleurotus ostreatus* (Korea), *P. ostreatus* (Taiwan), and *Pleurotus salmoneostramineus* contained the highest amounts of ergothioneine (2850.7, 1829.4, 1458.4, and 1245.0 mg/kg, respectively). Among mycelia, *Pleurotus eryngii* contained the highest amount of ergothioneine (1514.6 mg/kg). Overall, the species of the genus *Pleurotus* contained considerably large amounts of ergothioneine.

Our objective was to investigate the effect of cultivation conditions on mycelial biomass and ergothioneine content using one-factor-at-a-time methods. In addition, a central composite rotatable design (CCRD) was used to explore the interaction between precursor compositions. Response surface methodology was applied to optimize precursor composition for the enhancement of mycelial biomass and ergothioneine yield.

## II. MATERIALS AND METHODS

### A. Mushroom Material

The mycelium of *P. citrinopileatus* was obtained from Q-Yo Bio-Technology Farm, Pusin, Chunghua Country, Taiwan. The mycelia were grown on potato dextrose agar plates for 7 days at 25°C and then maintained at 4°C. For the production of mycelia, the culture was inoculated with 5% of inoculum into 250-mL flasks containing 100 mL of basal medium and incubated at 25°C and 125 rpm. The basal medium contained the following (1 L): 20 g glucose, 5 g yeast extract, 2 g (NH₄)₂SO₄, 0.5 g KH₂PO₄, 0.5 g K₂HPO₄, and 0.5 g MgSO₄·7H₂O.

The DNA was extracted from *P. citrinopileatus* mycelium using the commercial Plant Genomic DNA Purification Kit (GeneMark, Taipei, Taiwan) according to the manufacturer’s instructions. The DNA product was mixed with PCR Dye Master Mix II (GeneMark), containing 0.75 U of Taq DNA polymerase, reaction buffer, 2 mmol/L MgCl₂, 250 μmol/L dNTP, and enzyme stabilizer. The primers for amplification of DNA were internal transcribed spacer 1, TCCGTAGGTGAACCTGCGG, and internal transcribed spacer 4, TCTCCGGCTTATTGATATGC. The DNA product was amplified by a polymerase chain reaction gradient thermal cycler (Genesis 96; Pebio Scientific Co., Taipei, Taiwan); the sequence of the polymerase chain reaction product obtained is shown in Fig. 1. When the sequence was compared with the published sequences in GenBank (National Center for Biotechnology Information, Bethesda, MD), the first 11 sequences with the most identity were all *P. citrinopileatus* (Table 1). Furthermore, the first 2 sequences with a maximum score of 1136, query coverage of 95%, and a maximum identity of 99% were *P. citrinopileatus* JN234853.1 and HM561987.1.

### B. Growth Curve in Submerged Culture

The growth curves of mycelia in different media were studied. The culture was inoculated with 5% of inoculum (5 mL) in a 250-mL flask containing 95 mL of the basal medium (as described earlier) or mineral medium and incubated at 25°C and 125 rpm. The mineral medium contained the following (1 L): 20 g glucose, 5 g yeast extract, 0.068 g K₂HPO₄, and 100 mL mineral salt solution (2.3 g MgSO₄·7H₂O, 0.1 g FeSO₄·7H₂O, 0.023 g MnSO₄·H₂O, 0.06 g ZnSO₄·7H₂O, 0.08 g CaCl₂·2H₂O, and 0.03 g CuSO₄·5H₂O per L). During mycelial growth in submerged culture, an aliquot (3 mL) of the culture was withdrawn routinely and analyzed for biomass, residual sugar, and ergothioneine contents.
C. Optimization of Culture Conditions

The optimal culture conditions for mycelia with the highest ergothioneine content in the basal medium were created using 3 different methods. First, the one-factor-at-a-time method was studied for 5 factors: (1) temperature (20, 25, and 30°C); (2) inoculation ratio (1%, 5%, 10%, 15%, and 20%); (3) initial pH value adjusted with 3 N hydrochloric acid or sodium hydroxide solution (pH 2, 4, 8, 10, and 12) and control (pH 6.3–6.7); (4) supplemented 2% carbon source (20 g/L; glucose as the control, fructose, lactose, maltose, or sucrose); and (5) supplemented 0.5% nitrogen source (5 g/L; yeast extract as the control, corn steep, malt extract, peptone, or tryptone). Second, 2-stage stimulation was studied for 2 factors: (1) various pH values and (2) amino acid precursor (cysteine, histidine, and methionine) added to the submerged culture at various days (days 0, 7, and 14). Finally, CCRD experiments were used and the variables were selected according to the preliminary results.

Three precursors were found to be significant in the biomass and ergothioneine content of *P. citrinopileatus* mycelia in submerged culture. Therefore, a CCRD consisting of 3 variables (cysteine, histidine, and methionine), with each at 5 different levels, was used to fully elucidate the response surface near optimal and to provide a basis for the second-order polynomial approximation to the true response (Table 2). The CCRD combined the vertices of the hypercube whose coordinates were given by 2^3 full designs to provide for the estimation of curvature of the model. The experimental design is shown in Table 3; in total, 20 experiments and 6 replicates for the central point (trials 15–20) were used for the estimation of pure error mean square.

Experiments were randomized to maximize the effects of unexplained variability in the observed responses as a result of extraneous factors. The trials were performed in triplicate using the inoculation of 5% (5 mL) into 250-mL flasks containing 95 mL of medium under the conditions of 25°C and 125 rpm for 22 days. The CCRD experimental results were fitted with a second-order polynomial equation using a multiple regression technique. The following equation was proposed for the response ($Y_1$ and $Y_2$):

$$Y_i = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3,$$

where $X$ is the coded independent variable; $Y_i$ is the predicted response for mycelial growth ($i = 1$) and ergothioneine content ($i = 2$); $a_0$ is the value of the fitted response at the center point of the design; and $a_p$, $a_{ii'}$, and $a_{ij}$ are the linear, quadratic, and cross-product terms, respectively. Furthermore, to deduce workable optimal conditions, a graphic technique was used by one fixed variable at a predetermined optimal condition. Responses were monitored and results were compared with model predictions.

D. Biomass and Ergothioneine Assay

The mycelial growth of *P. citrinopileatus* was expressed as dry cell weight. The mycelium was harvested at the end of the incubation, filtered through...
preweighed filter paper (Whatman No. 1), and dried to a constant weight at 50°C. Ergothioneine of mycelia was extracted and analyzed according to the methods described previously. Ergothioneine content was quantified by the calibration curve of the authentic compound.

**E. Statistical Analysis**

The fitness of the second-order model was expressed by the coefficient of determination ($R^2$), and its statistical significance was determined using an $F$ test. The significances of regression and validation were tested using a $t$ test. SAS software (version 8.0; SAS Institute Inc., Cary, NC) and Sigma Plot 2001 software (version 7.0; SPSS Inc., Chicago, IL) were used for regression and graphical analyses of the data, respectively. The suitability of the polynomial models for predicting the optimum response values was tested under the recommended optimal conditions. Both experimental response data were separately analyzed using SPSS software.

Every trial for the study of optimal culture conditions was conducted in triplicate, and each assay

**TABLE 1**: The First 15 Sequences That Have the Most Identity of the Published Sequence in the Gene Bank Compared with the Internal Transcribed Spacer Ribosomal DNA of *Pleurotus citrinopileatus*

<table>
<thead>
<tr>
<th>Accession Number</th>
<th>Strain</th>
<th>Maximum Score</th>
<th>Query Coverage (%)</th>
<th>E Value</th>
<th>Maximum Identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JN234853.1</td>
<td><em>P. citrinopileatus</em></td>
<td>1136</td>
<td>95</td>
<td>0.0</td>
<td>99</td>
</tr>
<tr>
<td>HM561987.1</td>
<td><em>P. citrinopileatus</em></td>
<td>1136</td>
<td>95</td>
<td>0.0</td>
<td>99</td>
</tr>
<tr>
<td>JN043316.1</td>
<td><em>P. citrinopileatus</em></td>
<td>1129</td>
<td>94</td>
<td>0.0</td>
<td>99</td>
</tr>
<tr>
<td>EU424285.1</td>
<td><em>P. citrinopileatus</em></td>
<td>1127</td>
<td>95</td>
<td>0.0</td>
<td>99</td>
</tr>
<tr>
<td>AY540319.1</td>
<td><em>P. citrinopileatus</em></td>
<td>1127</td>
<td>95</td>
<td>0.0</td>
<td>99</td>
</tr>
<tr>
<td>AY540318.1</td>
<td><em>P. citrinopileatus</em></td>
<td>1127</td>
<td>95</td>
<td>0.0</td>
<td>99</td>
</tr>
<tr>
<td>AY265852.1</td>
<td><em>P. citrinopileatus</em></td>
<td>1122</td>
<td>94</td>
<td>0.0</td>
<td>99</td>
</tr>
<tr>
<td>AY265816.1</td>
<td><em>P. citrinopileatus</em></td>
<td>1120</td>
<td>94</td>
<td>0.0</td>
<td>99</td>
</tr>
<tr>
<td>JF758882.1</td>
<td><em>P. citrinopileatus</em></td>
<td>1116</td>
<td>94</td>
<td>0.0</td>
<td>99</td>
</tr>
<tr>
<td>DQ077889.1</td>
<td><em>P. citrinopileatus</em></td>
<td>1110</td>
<td>94</td>
<td>0.0</td>
<td>99</td>
</tr>
<tr>
<td>AY696301.1</td>
<td><em>P. citrinopileatus</em></td>
<td>1098</td>
<td>94</td>
<td>0.0</td>
<td>99</td>
</tr>
<tr>
<td>DQ077880.1</td>
<td><em>Pleurotus sp.</em></td>
<td>1096</td>
<td>94</td>
<td>0.0</td>
<td>99</td>
</tr>
<tr>
<td>KC416182.1</td>
<td><em>Fungal sp.</em></td>
<td>1086</td>
<td>92</td>
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<td>99</td>
</tr>
<tr>
<td>AB115043.1</td>
<td><em>P. citrinopileatus</em></td>
<td>1068</td>
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<td>0.0</td>
<td>99</td>
</tr>
<tr>
<td>EU424298.2</td>
<td><em>P. euosmus</em></td>
<td>1051</td>
<td>95</td>
<td>0.0</td>
<td>97</td>
</tr>
</tbody>
</table>

**TABLE 2**: Independent Variables and Experimental Levels for the Central Composite Rotatable Design

<table>
<thead>
<tr>
<th>Variable (mmol/L)</th>
<th>Coded Symbol</th>
<th>Coded Level</th>
<th>+d*</th>
<th>+1</th>
<th>0</th>
<th>−1</th>
<th>−d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine</td>
<td>$X_1$</td>
<td>13.05</td>
<td>11.00</td>
<td>8.00</td>
<td>5.00</td>
<td>2.95</td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>$X_2$</td>
<td>7.36</td>
<td>6.00</td>
<td>4.00</td>
<td>2.00</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>$X_3$</td>
<td>1.00</td>
<td>0.80</td>
<td>0.50</td>
<td>0.20</td>
<td>0.00</td>
<td></td>
</tr>
</tbody>
</table>

*d = (N)1/4 = (8)1/4 = 1.682
Biomasses peaked in the basal medium (8.70 g/L) at day 14 and in the mineral medium (11.22 g/L) at day 6. Along with increased incubation time, the mycelial dry biomasses decreased in 2 kinds of media. This phenomenon might be the result of the autolysis that occurred in the center of mycelial pellet, as described elsewhere. However, the ergothioneine contents in mycelia from basal or mineral media reached a peak of 2.87 mg/g dry weight (dw) at day 22 (Fig. 2B) and 2.86 mg/g dw at day 17 (Fig. 2D), respectively. Since no difference was found in the ergothioneine content of mycelia from the 2 media, the basal medium was used for further studies.

Ergothioneine was a secondary metabolite; its accumulation was not affected by mycelial autolysis also was determined in triplicate. The experimental data were expressed as mean ± standard error and subjected to an analysis of variance for a completely random design to determine the Fisher’s least significant difference at the level of α = 0.05.

### III. RESULTS AND DISCUSSION

#### A. Growth Curve in Submerged Culture

As a result of mycelial growth, the residual sugar content in the shaken flask decreased quickly in basal and mineral media from days 4 to 12 (from 17.42 to 0.69 g/L; Fig. 2A) and from days 4 to 11 (from 17.22 to 0.40 g/L; Fig. 2C), respectively. The biomasses peaked in the basal medium (8.70 g/L) at day 14 and in the mineral medium (11.22 g/L) at day 6. Along with increased incubation time, the mycelial dry biomasses decreased in 2 kinds of media. This phenomenon might be the result of the autolysis that occurred in the center of mycelial pellet, as described elsewhere. However, the ergothioneine contents in mycelia from basal or mineral media reached a peak of 2.87 mg/g dry weight (dw) at day 22 (Fig. 2B) and 2.86 mg/g dw at day 17 (Fig. 2D), respectively. Since no difference was found in the ergothioneine content of mycelia from the 2 media, the basal medium was used for further studies.

Ergothioneine was a secondary metabolite; its accumulation was not affected by mycelial autolysis

#### TABLE 3: Central Composite Rotatable Design Responses in Biomass, Final pH of the Filtrate, and Ergothioneine content of Pleurotus citrinopileatus Mycelium in Submerged Cultivation

<table>
<thead>
<tr>
<th>Trial No.</th>
<th>Variable Level</th>
<th>Response*</th>
<th>Biomass (g/L)</th>
<th>Final pH</th>
<th>Ergothioneine Content mg/g Dry Weight</th>
<th>Ergothioneine Content mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+1 +1 +1</td>
<td></td>
<td>8.54 ± 0.36</td>
<td>7.36 ± 0.02</td>
<td>3.01 ± 0.04</td>
<td>25.71 ± 0.37</td>
</tr>
<tr>
<td>2</td>
<td>+1 +1 −1</td>
<td></td>
<td>7.91 ± 0.47</td>
<td>7.03 ± 0.07</td>
<td>2.85 ± 0.08</td>
<td>22.57 ± 0.67</td>
</tr>
<tr>
<td>3</td>
<td>+1 −1 +1</td>
<td></td>
<td>8.70 ± 0.35</td>
<td>6.82 ± 0.10</td>
<td>5.43 ± 0.43</td>
<td>47.24 ± 3.75</td>
</tr>
<tr>
<td>4</td>
<td>+1 −1 −1</td>
<td></td>
<td>8.21 ± 0.45</td>
<td>6.43 ± 0.10</td>
<td>5.98 ± 0.38</td>
<td>49.13 ± 3.09</td>
</tr>
<tr>
<td>5</td>
<td>−1 +1 +1</td>
<td></td>
<td>8.79 ± 0.16</td>
<td>7.23 ± 0.05</td>
<td>4.06 ± 0.26</td>
<td>35.71 ± 2.26</td>
</tr>
<tr>
<td>6</td>
<td>−1 +1 −1</td>
<td></td>
<td>8.55 ± 0.20</td>
<td>7.13 ± 0.61</td>
<td>2.02 ± 0.06</td>
<td>17.25 ± 0.54</td>
</tr>
<tr>
<td>7</td>
<td>−1 −1 +1</td>
<td></td>
<td>6.96 ± 0.25</td>
<td>7.15 ± 0.04</td>
<td>5.06 ± 0.04</td>
<td>35.20 ± 0.31</td>
</tr>
<tr>
<td>8</td>
<td>−1 −1 −1</td>
<td></td>
<td>7.45 ± 0.11</td>
<td>6.55 ± 0.10</td>
<td>5.26 ± 0.07</td>
<td>39.17 ± 0.49</td>
</tr>
<tr>
<td>9</td>
<td>+d 0 0</td>
<td></td>
<td>8.94 ± 0.64</td>
<td>7.58 ± 0.01</td>
<td>4.32 ± 0.03</td>
<td>38.67 ± 0.31</td>
</tr>
<tr>
<td>10</td>
<td>−d 0 0</td>
<td></td>
<td>9.89 ± 0.03</td>
<td>7.11 ± 0.26</td>
<td>4.30 ± 0.03</td>
<td>42.51 ± 0.29</td>
</tr>
<tr>
<td>11</td>
<td>0 +d 0</td>
<td></td>
<td>10.15 ± 0.10</td>
<td>7.09 ± 0.59</td>
<td>1.15 ± 0.03</td>
<td>11.67 ± 0.25</td>
</tr>
<tr>
<td>12</td>
<td>0 −d 0</td>
<td></td>
<td>7.11 ± 0.05</td>
<td>6.32 ± 0.06</td>
<td>5.03 ± 0.11</td>
<td>35.79 ± 0.82</td>
</tr>
<tr>
<td>13</td>
<td>0 0 +d</td>
<td></td>
<td>8.35 ± 0.68</td>
<td>7.08 ± 0.28</td>
<td>4.84 ± 0.18</td>
<td>40.40 ± 1.51</td>
</tr>
<tr>
<td>14</td>
<td>0 0 −d</td>
<td></td>
<td>8.87 ± 0.02</td>
<td>7.05 ± 0.21</td>
<td>4.97 ± 0.97</td>
<td>44.13 ± 3.11</td>
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<tr>
<td>15</td>
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<td></td>
<td>7.37 ± 0.02</td>
<td>7.51 ± 0.05</td>
<td>14.67 ± 0.32</td>
<td>113.41 ± 4.96</td>
</tr>
<tr>
<td>16</td>
<td>0 0 0</td>
<td></td>
<td>6.94 ± 0.13</td>
<td>7.37 ± 0.01</td>
<td>14.56 ± 0.08</td>
<td>101.04 ± 3.24</td>
</tr>
<tr>
<td>17</td>
<td>0 0 0</td>
<td></td>
<td>6.78 ± 0.50</td>
<td>7.95 ± 0.01</td>
<td>13.55 ± 0.09</td>
<td>107.05 ± 4.29</td>
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<tr>
<td>18</td>
<td>0 0 0</td>
<td></td>
<td>7.90 ± 0.08</td>
<td>7.53 ± 0.10</td>
<td>12.91 ± 1.12</td>
<td>85.18 ± 2.64</td>
</tr>
<tr>
<td>19</td>
<td>0 0 0</td>
<td></td>
<td>6.60 ± 0.06</td>
<td>7.82 ± 0.03</td>
<td>14.61 ± 0.98</td>
<td>99.09 ± 3.67</td>
</tr>
<tr>
<td>20</td>
<td>0 0 0</td>
<td></td>
<td>6.71 ± 0.21</td>
<td>7.81 ± 0.04</td>
<td>13.23 ± 0.44</td>
<td>88.76 ± 5.31</td>
</tr>
</tbody>
</table>

*The responses were achieved after being incubated for 22 days.
and peaked several days after the maximum biomass was obtained. During incubation, the ergothioneine content and the pH value consistently increased over time. Surprisingly, the incubation time at which the ergothioneine content (milligrams per gram dw or milligrams per liter) reached the maximum is coincidentally the time at which the highest pH value was detected. It seems that ergothioneine production prefers a higher pH value, especially an alkaline pH.

B. One Factor at a Time

According to the preliminary studies, the culture was inoculated at the ratio of 5% and incubated at 25°C and 125 rpm, and the mycelia harvested at day 22 showed the highest ergothioneine content. Therefore, in the following one-factor-at-a-time studies, mycelia were harvested at day 22. The culture incubated at 25°C showed the largest amount of biomass (7.59 g/L) and ergothioneine (1.81 mg/g dw and 13.75 mg/L), better than those incubated at 20°C and 30°C (Table 4). Higher inoculation ratios (15–20%) gave rise to higher ergothioneine contents (2.02–2.01 mg/g dw) in mycelia, but the biomasses decreased (3.85–3.96 g/L). Therefore, the inoculation ratio for further study was 5% (5 mL).

The culture with an initial pH of 10 showed the most biomass and highest ergothioneine content (Table 4). Glucose as the control carbon source showed the most biomass and the second highest ergothioneine content (1.81 mg/g dw). Maltose as the carbon source showed the second largest amount of biomass but the highest ergothioneine content (2.03 mg/g dw). When considering the ergothioneine yield (grams per liter), the optimal carbon source used for mycelial growth was glucose. Yeast extract as the control nitrogen source showed the most biomass, but tryptone resulted in mycelia with

FIG. 2: Biomass, pH of the filtrate, residual sugar, and ergothioneine in Pleurotus citrinopileatus mycelium from submerged cultivation with basal medium (A, B) and mineral medium (C, D).
the highest ergothioneine content. Using corn steep as the nitrogen source, however, created no ergothioneine content.

Similarly, Liang et al.\textsuperscript{22} tried to enhance ergothioneine production in submerged cultivation of *P. eryngii* and found that tryptone gave rise to the highest ergothioneine content. The amounts of precursors—cysteine, histidine, and methionine—for the synthesis of ergothioneine in yeast extract were reported to be 0.2\%, 1.2\%, and 0.8\%, respectively,
whereas those in tryptone were 0.4%, 1.5%, and 1.6%, respectively. Therefore, we speculate that the highest ergothioneine content in the mycelia grown in the medium with tryptone as the nitrogen source was the result of its larger amounts of precursors.

Using a one-factor-at-a-time method for 22 days of incubation, the optimal culture conditions for mycelia with the highest ergothioneine content were determined: incubation temperature, 25°C; inoculation ratio, 5%; initial pH, 10; 2% glucose; and 0.5% yeast extract.

C. Two-Stage Stimulation

According to the one-factor-at-a-time studies, the initial pH of the basal medium was adjusted to 10 to obtain the highest ergothioneine content. Based on the submerged growth curve of the basal medium (Fig. 2A), the incubation period could be separated into 4 stages: the lag stage at days 0–2, the exponential stage at days 2–6, the stationary stage at days 6–14, and the death stage at days 14–24. Therefore, the pH of the culture was changed at day 0, 7, or 14 and the culture with the pH unchanged was used as the control. Both the most biomass and highest ergothioneine content were found in the culture with the pH changed at day 0 (Fig. 3). Since ergothioneine does not auto-oxidize at a physiological pH, it is very stable in aqueous solutions. This research found that ergothioneine was also very stable in the alkaline solution.

Askari and Melville mentioned that ergothioneine could be biosynthesized by fungi using 3 amino acids as precursors: histidine, cysteine, and methionine. Melville et al. noted that histidine contained an imidazole ring, which contributed to the molecular structure of ergothioneine, and that cysteine and methionine could provide sulphydryl and methyl groups, respectively. Therefore, the culture was added with 4 mmol/L of the respective precursor at day 0, 7, or 14 and incubated under the optimal conditions described above.

Mycelial growth was inhibited when the respective precursor was added at days 0, 7, and 14, as evidenced by less biomass in the culture with precursor added (5.17–6.49 g/L) than in the control (7.59 g/L; Fig. 4). On the contrary, the ergothioneine content in the culture with precursor added (2.82–16.31 mg/g dw) was higher than that of the control (1.81 mg/g dw). Interestingly, the ergothioneine content in the culture with the respective precursors added at day 7 was higher than that in the culture with the precursor added at day 0 or 14. However, the ergothioneine content was highest (16.31 mg/g dw) in the culture with 4 mmol/L cysteine added at day 7.

Taking the ergothioneine content of the control as 100% (1.81 mg/g dw), the content in the culture with cysteine added at days 0, 7, and 14 was 480%, 901%, and 473%, respectively. Ergothioneine in the culture with histidine added at days 0, 7, and 14 was 471%, 507%, and 492%, respectively, and that in
the culture with methionine added at days 0, 7, and 14 was 156%, 367%, and 256%, respectively. It is obvious that the addition of precursors effectively increased the ergothioneine content, and cysteine did so most effectively. In addition, the addition of precursor at day 7 showed higher ergothioneine content than addition at days 0 and 14.

Lee et al.\textsuperscript{25} suggested that the addition of methionine to \textit{Ganoderma neo-japonicum} culture is an efficient way to enhance ergothioneine production, but mycelia growth was inhibited at 16 mmol/L methionine. However, at 4 mmol/L methionine, the ergothioneine content of mycelia was effectively increased. Liang et al.\textsuperscript{22} reported that with 4 mmol/L histidine or amino acid mix added, biomass and ergothioneine content of \textit{P. eryngii} mycelia were higher than those of the control. Summarily, higher ergothioneine content in mycelia could be achieved by means of optimal culture conditions and the addition of precursors.

**D. Response Surface Methodology**

Lee et al.\textsuperscript{25} found that the addition of 2 mmol/L histidine, 2 mmol/L cysteine, and 2 mmol/L methionine resulted in higher ergothioneine content in \textit{G. neo-japonicum} mycelia than the addition of a single amino acid. Liang et al.\textsuperscript{22} showed that the ergothioneine content of mycelia with 4 mmol/L histidine added at day 7 was 185% (3.10 mg/g) when that of the control was taken as 100% (1.68 mg/g), whereas that of that of mycelia with amino acid mix (4 mmol/L histidine, 1 mmol/L cysteine, and 1 mmol/L methionine) added at day 5 was 280% (4.70 mg/g). Since amino acid
mix contained 3 precursors, higher ergothioneine content was expected. However, \textit{P. citrinopileatus} and \textit{P. eryngii} were used in the research and by Liang et al.,\textsuperscript{22} respectively. It reveals that ergothioneine production in different species might need different precursors and different combinations.

To optimize the production of ergothioneine, 3 variables obtained from the previous one-factor-at-a-time experiments were investigated using the CCRD statistical approach, which could identify and quantify interactions between variables. Considerable variation was observed in the results of biomass, pH, and ergothioneine content (Table 3). The ergothioneine content ranged from 1.15 to 14.67 mg/g dw; trials 11 and 15 had the minimum and maximum amounts of ergothioneine, respectively. Ergothioneine content as the center of the design (trials 15–20) was 12.91–14.67 mg/g dw, much higher than that of trials 1–14 (1.15–5.98 mg/g dw).

Analysis of variance was conducted to assess the significant effects of independent variables on the response, which was notably affected by the various treatment combinations. In the submerged fermentation of \textit{P. citrinopileatus} mycelium, the 3 precursors had significant effects on mycelial ergothioneine content at the 0.1% level. Analysis of variance also was performed to determine the lack of fit and the significance of the linear, quadratic, and cross-product effects of independent variables on the response. The lack-of-fit test was a measure of the failure of a model to represent data in the experimental domain at which points were not included in the regression.\textsuperscript{26}

The polynomial model for ergothioneine content was significant at a 0.1% level. More specifically, both the linear and quadratic effects for mycelial ergothioneine content were significant at a 0.1% level. However, the cross-product effect was not significant. The \( R^2 \) value for ergothioneine content was 0.9880. In other words, a high correlation coefficient \((r = 0.994)\) was obtained for the significant model of ergothioneine production with an insignificant lack-of-fit variation. The coefficients of variation for the model were within the acceptable range \((<10.12\%)\). Therefore, the response surface model developed was desirable.

The following overall second-order polynomial equation for the response \((Y = \text{ergothioneine content})\) was found:

\[
y = 13.90*** + 0.07X_1 - 1.19X_2*** + 0.09X_3 - 3.25X_1^2*** - 3.68X_2^2 - 3.04X_3^2*** - 0.16X_1X_2 - 0.28X_1X_3 + 0.37X_2X_3
\]

The intercept, histidine \((X_2)\), and quadratic terms \((X_1^2\) and \(X_2^2\)) for ergothioneine were significant at the 0.1% level (***)). Negative coefficients of \(X_1\) indicated a linear effect to decrease the ergothioneine yield. In addition, negative coefficients of the quadratic terms \((X_1^2\) and \(X_2^2\)) had negative effects on the ergothioneine yield. However, coefficients of cysteine \((X_1)\), methionine \((X_3)\), and cross-product terms \((X_1X_2, X_1X_3, \text{and } X_2X_3)\) were low and insignificant.

The contour plot and response surface curve of the ergothioneine content was downward hemisphere and simulated as functions of cysteine \((X_1)\) versus methionine \((X_3)\) at one fixed variable \((4 \text{ mmol/L histidine, coded as level 0})\) since the histidine \((X_2)\) was significant (Fig. 5). Several concentric circles with 12 mg/g dw intervals were found for the ergothioneine content (data not shown). The coded levels of the center were all calculated to be 0 for cysteine, histidine, and methionine.

The actual levels of the center, however, were 8, 4, and 0.5 mmol/L for cysteine \((X_1)\), histidine \((X_2)\), and methionine, respectively. Under the conditions described above, the predicted ergothioneine yields would be increased to 13.90 mg/g dw. The suitability of the polynomial models for predicting the optimal response value was tested under the recommended combination of optimal conditions. The maximal ergothioneine content of mycelia was 14.57 mg/g dw. However, the predicted and experimental ergothioneine content were the same at the 95% confidence level. It is obvious that the optimization of ergothioneine content by \textit{P. citrinopileatus} in submerged fermentation was established.

The mycelial biomass values ranged from 6.60 to 10.15 g/L; trials 19 and 11 had the smallest and largest biomasses, respectively. The mycelial biomass values as the center of the design (trials 15–20) were 6.60–7.90 mg/g dw, lower than those of trials...
1–14 (6.96–1015 mg/g dw). The following overall second-order polynomial equation for the response ($Y$ = mycelial biomass) was found:

$$Y = 7.80*** + 0.00X_1 + 0.56X_2 + 0.00X_3 + 0.66X_1^2 + 0.38X_2^2 + 0.37X_3^2 - 0.42X_1X_2 - 0.17X_1X_3 + 0.11X_2X_3$$

Only the intercept for biomass was significant at the 0.1% level. However, coefficients of cysteine ($X_1$), histidine ($X_2$), methionine ($X_3$), quadratic terms ($X_1^2$, $X_2^2$, and $X_3^2$), and cross-product terms ($X_1X_2$, $X_1X_3$, and $X_2X_3$) were low and insignificant.

The contour plot and response surface curve of the biomass was inversely upward hemisphere and simulated as functions of cysteine ($X_1$) versus methionine ($X_3$) at one fixed variable (4 mmol/L histidine, coded as level 0) since the histidine ($X_2$) was significant (Fig. 5). Several concentric circles with 7.5 g/L intervals were found for mycelia (data not shown). The coded levels of the center were all calculated to be 0 for cysteine, histidine, and methionine, respectively. Comparing the ergothioneine content with biomass of *P. citrinopileatus* mycelia, we found that the ergothioneine content and mycelial biomass correlated inversely. In other words, the ergothioneine content at the highest point was the biomass at its lowest point.

### E. Optimal Harvest Day under Various Submerged Conditions

According to the preliminary studies, the culture in various media (mineral, basal, or tryptone media) with a 5% inoculation rate was incubated at 25°C and 125 rpm; the growth curve was followed to harvest the mycelia on the day when the most ergothioneine was found. The mycelia harvested at day 17 from mineral medium without pH adjusted or precursor added showed ergothioneine content of 13.26 mg/L (Table 5). The mycelia harvested at day 22 from basal medium without pH adjusted or precursor added showed ergothioneine content of 18.17 mg/L. However, on the basis of mycelial dw, the ergothioneine contents of mycelia harvested at day 17 from mineral medium and at day 22 from basal medium were similar.

According to the one-factor-at-a-time studies, the mycelia harvested at day 22 from tryptone medium (tryptone as the nitrogen source in basal medium) showed ergothioneine content of 34.58 mg/L. With the addition of 4 mmol/L cysteine at day 7, the mycelia harvested at day 22 showed ergothioneine content of 86.39 mg/L (475%) when that of the control was taken as 100% (18.17 mg/g). In addition, with adjustment of pH to 10 at day 0,
the mycelia harvested at day 22 showed ergothioneine content of 88.49 mg/L (487%). However, with adjustment of pH to 10 at day 0, the mycelia harvested at day 16 showed ergothioneine content of 35.67 mg/L (196%), whereas that harvested at day 12 showed higher ergothioneine content than the control. Finally, with the addition of a complex amino acid solution at day 7, the mycelia harvested at day 16 showed ergothioneine content of 97.69 mg/L (538%), whereas that harvested at day 10 showed higher ergothioneine content than the control. These results indicate that the addition of precursors not only increases the ergothioneine content but also shortens the submerged fermentation time of P. citrinopileatus mycelia.

IV. CONCLUSIONS

Submerged culture was studied to produce P. citrinopileatus mycelia with high ergothioneine content using a one-factor-at-a-time, two-stage stimulation and CCRD method. The optimal culture conditions for mycelia harvested at day 22 were a temperature of 25°C, an inoculation rate of 5%, 2% glucose, 0.5% yeast extract, and an initial pH of 10. With the addition of cysteine or a complex amino acid solution at day 7, the biomass and ergothioneine content of mycelia were higher than those of the control produced under optimal culture conditions. The results obtained from response surface methodology showed that the recommended combination for cysteine, histidine, and methionine were 8, 4, and 0.5 mmol/L, respectively. With the addition of complex precursors and under the optimal culture conditions, the ergothioneine content of mycelia harvested at days 16–20 could be higher than that of the control. The results from this research are applicable in the cultivation of mycelia with high ergothioneine production.

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REFERENCES