Process Development of Ergothioneine by Submerged Fermentation of a Novel Medicinal Mushroom Panus Conchatus

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Abstract

Ergothioneine is a natural and safe antioxidant playing an important role in anti-aging and the prevention of various diseases. Mushrooms are the main dietary source of ergothioneine. This study aims to report a kind of medicinal mushroom *Panus conchatus* with great potential for the bioproduction of ergothioneine. Molasses and soy peptone could promote cell growth of *Panus conchatus* and enhance the accumulation of ergothioneine. Meanwhile, three precursors of cysteine, histidine and methionine were added to the broth, and the highest ergothioneine concentration of 148.79mg/L was obtained when adding 0.4 g/L cysteine. Finally, the crude ergothioneine extract was purified and further evaluated. The ergothioneine from *Panus conchatus* showed higher antioxidant activity than vitamin C and glutathione with good stability at lower pH environment. This research would provide a new method for the bioproduction of ergothioneine and lay foundation for the in-depth study about the exploration of natural products from *Panus conchatus*.

1. Introduction

Ergothioneine (EGT) is a sulfur-containing histidine derivative first discovered in ergot fungi in 1909. It has two forms of tautomeric structure, thione and thiol, but mainly exists in the form of thione at physiological pH (Hartman 1990). EGT is a powerful antioxidant, an excellent scavenger of reactive oxygen species (ROS) and an inhibitor of lipid peroxides (Chaves et al. 2019; Pahila et al. 2017). It has been linked to the improvement of a variety of diseases, including cancer, inflammation, depression and neurological diseases (Yoshida et al. 2019; Cheah et al. 2017; Cao et al. 2020; Nakamichi et al. 2016; Nishida et al. 2018; Tyler et al. 2020). Human beings cannot synthesize EGT directly, but EGT can be ingested through diet (Han et al. 2021). EGT entering the human body would be accumulated in various parts of the body, such as liver, kidney and other organs (Latner 1948; Ohara et al. 1952) through specific transporters (Grundemann et al. 2005; Gruendemann 2012). Due to the superior health benefits of EGT, this compound has been widely used as a natural antioxidant in the food and cosmetics industries.

Chemical synthesis, extraction and microbial fermentation were three common-used methods for EGT production. Microbial fermentation has attracted wide attention because of its higher productivity, safer production process and lower cost. Bacteria, fungi, yeast, cyanobacteria, actinomycetes and plants can be used as the producers of EGT (Fujitani, Alamgir, and Tani 2018; Pfeiffer et al. 2011). Previous studies have shown that mushrooms can accumulate high levels of EGT, such as *Pleurotus eryngii*, *Lentinus edodes*, *Pleurotus ostreatus*, *Agaricus bisporus*, *Pleutotus citrinopileatus*, *Cantharellus cibarius* and *Boletus edulis*, especially *Boletus edulis*, up to 7.27mg/g dry weight (Kalaras et al. 2017; Chen, Ho, Liang, et al. 2012; Tepwong et al. 2012; Chen, Ho, Hsieh, et al. 2012). In the traditional mushroom cultivation, EGT was usually harvested on the 37-45 days, and the cultivation cycle was long, which was not suitable for the industrial production of EGT (Estrada et al. 2009). However, the establishment of the deep fermentation technology of mushroom mycelium could break the deadlock, shorten the fermentation time, and provide a bridge for the commercial production of EGT.

Until now, people have tried different strategies to improve the cell growth and EGT production in the macrofungal fermentation, including optimizing carbon and nitrogen source, adjusting temperature and harvest time. Tepwong et al. (Tepwong et al. 2012) demonstrated that monosaccharides and most amino acids could promote EGT secretion from edible mycelium of *Lentinus edodes*, and the combination of fructose and aspartic acid increased EGT production by 3.15-fold. Liang et al. (Liang et al. 2013) found that the optimal temperature for the growth of *Pleurotus eryngii* mycelium was different from that for EGT synthesis. When Lin et al. (Lin et al. 2015) cultured *Pleurotus citrinopileatus* in mineral medium containing amino acids, the EGT production of mycelia increased by 43.27% compared with the control, respectively. However, most of the study focused on the strain belonging to the genes of *Pleurotus*. In order to break through technological monopoly and renew patents, it was still necessary to develop new strains with potential of high EGT yield.

In this study, a kind of medicinal mushroom of *Panus conchatus* was isolated and found to produce EGT. Ergothioneine productivity of *Panus conchatus* was determined with various media components. In addition, three precursors of cysteine, histidine and methionine were added to enhance ergothioneine accumulation. Furthermore, the crude ergothioneine extract was purified and further evaluated. This study reported the fermentation characteristics of a new strain of *Panus conchatus* for EGT production, which will provide valuable experimental basis for the future production of EGT by submerged fermentation of mycelium.

2. Materials And Methods

2.1 Stains and growth conditions

Panus conchatus were stored at 4℃ in a refrigerator in our lab of Nanjing Tech University. The stored strain was transferred to the PDA plate and cultivated twice at 25°C until the hyphae overwhelm the entire plate. 10 pieces of mycelium were picked from the plate and transferred to the seed medium (26g/L potatoes dextrose water, 2g/L peptone, 2g/L KH_2PO_4, 2g/L $MgSO_4{\cdot}7H_2O$), and cultivated at 25℃ for 72h with shaking at 150rpm. Then, the seed liquid was inoculated with 10% (v/v) of the volume ratio into the fermentation medium (30g/L glucose, 3g/L soybean meal, 2g/L yeast extract, 0.8g/L KH_2PO_4, 0.7g/L $MgSO_4{\cdot}7H_2O$) and cultured at 25℃ for 168h with shaking at 150rpm.

2.2 Determination of dry cell weight and substrate consumption

Fermentation liquid was removed by filtration, and the dry cell weight was measured after drying the wet fungus in an oven at 65℃ to a constant weight. The residual sugars concentration in culture medium was determined by biosensor SBA-40C. The content of glycerol in the medium was titrated by NaOH. 2mL fermentation broth was centrifugated at 8000rpm for 5min and 1mL supernatant was mixed with 25mL 0.1m $NaIO_4$ for 15min, then 5ml of 50% $(CH_2OH)_2$ was added and mixed evenly for 20min. After that, 1-2 drops of phenolphthalein indicator were added and titrated with 0.1m NaOH until the solution turned pink. The glycerol concentration was calculated as following: $C_{glycerol} = V_{NaOH} \times C_{NaOH} \times 9.21$.

2.3 Genotypic identification and phenotypic identification of Panus conchatus

Strains were identified by morphology and 18SrDNA sequencing. A light microscope (a, LEICA DM1000, 40 × magnification) was used to observe the morphology of the hyphae. The fungal DNA extraction kit (Omega Bio-Tek) was used to isolate and identify the genomic DNA of the strain. Fungal ITS primers ITS1 (5 '-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5 '-TCCTCCGCTTATTGATATGC-3') were used to amplify the gene encoding 18SrDNA. The amplified products were purified by gel electrophoresis with a gel extraction kit and sent to General Biotech for sequencing. Homology analysis was conducted to identify the strains by BLAST tool in NCBI.

2.4 Optimization of cultivation medium and environmental conditions

Seven carbon sources of molasses, fructose, sucrose, maltose, glucose, glycerol and dextrin and six nitrogen sources of peptone, soybean peptone, casein peptone, soybean meal powder, beef extract and yeast powder were used to cultivated *Panus conchatus* and determine the final EGT productivity. 50g/L of glycerol and 30g/L of soybean peptone were used as the corresponding carbon sources and nitrogen sources respectively. Four different rotation speed of 60, 90, 120 and 150rpm was used to further study the effect of different oxygen supply conditions on EGT production. Three amino acids of histidine, methionine and cysteine were used as the precursors to enhance EGT production at five different concentrations (0.1, 0.2, 0.3, 0.4 and 0.5g/L).

2.5 EGT extraction and analysis

EGT was extracted by high temperature crushing method. 50mL of fermentation broth was collected and centrifuged to remove the fermentation liquid, then washed for three times by water. The final liquid was kept in a constant volume to 50mL and bathed in water at 95℃ for 1h. Then, the treated samples were centrifuged and the supernatant was collected for further analysis. High performance liquid chromatography system (Thermo Fisher Scientific, China) was used to measure EGT. UltiMate 3000 was determined at wavelength of 254nm by using a photon diode array UV detector. Accucore C18 (Dimension, 150×4.6, USA) was operated at 25℃ using 5% methanol (Adjust pH5.0 with H_3BO_3.) at a flow rate of 0.7 mL/min to measure the concentration of EGT.

2.6 Activity evaluation and stability analysis of ergothioneine

To evaluate the antioxidant activity of the crude EGT extract (EGTEX), glutathione (GSH) and Vitamin C (VC) were also detected as the control by the DPPH assay (Szabo et al. 2007). 15.8 mg DPPH was mixed with 200mL anhydrous ethanol to produce a 2×10^{-4}M DPPH-ethanol solution which stored at 2-8°C in the dark and used within 3.5h. Five different concentrations (50, 100, 200, 500 and 1000mg/L) of EGTEX, GSH, and VC samples were prepared. 1mL anhydrous ethanol solution and 3mL DPPH solution were mixed and reacted darkly for 30min as the control group A_0. 1mL sample solution and 3mL DPPH solution were mixed and reacted in dark for 30min as experimental group A_1. The blank group A_2 was mixed with 1mL sample solution and 3mL anhydrous ethanol solution for 30min. Then the absorbance of each group was measured at 517nm, and the DPPH radical scavenging capacity was calculated by the following formula: $DPPH = \frac{A_0-(A_1-A_2)}{A_0} \times 100\%$

Eight different temperatures (25, 35, 45, 55, 65, 75, 85 and 95℃) and ten different pH value (2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0) were used to detect the antioxidant activity of 100mg/L EGT. 10 tubes of 8mL EGT solution were held at different temperatures for 30min and then the DPPH clearance was measured by the above method. Ten tubes of 10 ml EGT solution were adjusted with 1mol/L NaOH and HCl to ten kinds of pH and then DPPH clearance was determined by the above method.

3. Results And Discussion

3.1 Genotypic identification and phenotypic identification of Panus conchatus

The medicinal fungus of *Panus conchatus* was firstly cultivated in the PDA plate. Colonies appeared to be white through 7 days cultivation (Fig. 1a) and its mycelium showed thick and robust with some lumps under the light microscope (Fig. 1c). Results of 18SrDNA sequencing results showed that this strain shared 98% sequence similarity with *Panus conchatus*. Then the strain was cultivated in the initial liquid medium to detect the ability for EGT production. The retention time of the product was 5.3 min corresponding to the position of standard substrate of EGT (Fig. 1d) and the initial titer could reach 19.35mg/L, indicating good potential for EGT production. It was found that it could produce EGT and the initial yield was 19.35mg/L.

3.2 Choosing the optimal nitrogen sources

In order to further improve the yield of EGT, we investigated the effects of different carbon and nitrogen sources on its fermentation performance. Six organic nitrogen sources, including peptone, soy peptone, casein peptone, soybean meal powder, beef extract and yeast powder were selected to investigate the effect on the biosynthesis of EGT in *Panus conchatus*.

As shown in Table 1, soy peptone group has the highest glycerol consumption rate of 0.38g/L/h and the highest EGT yield of 40.13mg/L, indicating that, soy peptone could be effectively used by *Panus conchatus* to synthesize EGT. However, when using beef extract as the nitrogen source, the glycerol consumption rate was nearly to the highest value, but its EGT yield only reached the half of the soybean peptone group. These results indicated that beef extract was a good nitrogen source nitrogen source and could promote the growth of *Panus conchatus* but could not stimulate the accumulation of EGT. In addition, peptone was the best nitrogen source for cell growth of *Panus conchatus* with a dry weight of 21.66g/L, followed by yeast powder and beef extract. As we all know, the main components of soy peptone were derived from plant protein, indicating that the hydrolysate of animal protein was more suitable for cell growth of *Panus conchatus*.

Table 1
Effect of different kinds of nitrogen sources on EGT production

Nitrogen source	Glycerol consumption rate	Dry cell weight	EGT yield	Fermentation time	EGT productivity
	g/L/h	g/L	mg/L	h	mg/L/h
Peptone	0.22±0.016	21.66±2.72	20.22±1.76	192	0.11
Soy peptone	0.38±0.011	16.08±1.13	40.13±3.89	144	0.28
Casein peptone	0.23±0.019	10.16±1.26	33.41±2.23	240	0.14
Soybean meal powder	0.27±0.027	-	19.90±1.76	240	0.08
Beef extract	0.37±0.019	16.74±1.50	21.33±1.17	96	0.22
Yeast powder	0.24±0.020	17.50±2.26	36.64±0.98	240	0.15
* '-' biomass could not be measured due to the soybean meal powder is solid particles.					

In terms of promoting product synthesis, soy peptone and yeast powder were more effective. The yield of EGT reached 40.13 mg/L and 36.64 mg/L, respectively, and the unit yield of peptone reached 0.28 mg/h. Soy peptone is a dry powder prepared by special hydrolysis of soybean as a substrate. It is rich in lipid, dietary fiber and various trace elements. Beef extract does not contain dietary fiber, and soybean meal powder contains a very small amount of lipid, so lipid and dietary fiber may be the reason that promotes the synthesis of EGT. In addition, different nitrogen sources also produced different morphology of mycelium (Fig. 2). The morphology of mycelium also has great influence on the rheological properties of fermentation broth (Riley et al. 2000). Cells in yeast powder and soy peptone were yellow and spherical with larger particle diameters. In peptone and beef extract, it is yellow and small in size and spherical. In casein peptone and soybean meal powder, white hyphae are mostly present. Combing with the results of final EGT yield, it seems the large particle size microspheres were more likely to promote the synthesis of EGT. This conclusion was also confirmed in the fermentation of *Lentinus edodes* (Tepwong, Giri, and Ohshima 2012).

3.3 Choosing the optimal carbon sources

Carbon source could provide the main energy source for cell growth and construct the main component of cytoskeleton. Fructose, glucose, sucrose, maltose, glycerin, dextrin and molasses were compared in this study to investigate the effect of different carbon sources on the fermentation performance of *Panus conchatus*. As shown in Table 2, glucose was the best carbon source for cell growth and cell dry weight reached the maximum value of 13.12 g/L at 96h. The cell dry weight when using maltose as the carbon source was also near the maximum value but the fermentation time extended to 192h, indicating the maltose could not be used effectively by *Panus conchatus*. Similar results were also observed when using the polysaccharide like dextrin or disaccharides like sucrose as the carbon source. Further

comparing the morphology (Fig. 2), we found glucose induced cell to be mycelium while cell formed the pellets with relative larger size in maltose group.

Table 2
Effect of different kinds of carbon sources on EGT production

Carbon source	Dry cell weight	EGT yield	Fermentation time	EGT productivity
	g/L	mg/L	h	mg/L/h
Molasses	9.70±0.54	81.44±5.01	96	0.85
Fructose	7.93±0.55	22.69±1.99	144	0.16
Sucrose	8.65±0.70	11.75±0.57	192	0.06
Maltose	11.37±0.91	14.74±1.11	192	0.08
Glucose	13.12±0.76	18.78±1.67	96	0.20
Glycerol	9.74±0.41	33.35±1.12	96	0.35
Dextrin	-	7.20±0.88	192	0.04
* '-' biomass could not be measured due to the dextrin is slightly soluble in water.				

Moreover, the highest EGT yield of 81.44 mg/L was obtained when using the molasses as carbon source, which was 2.44 times and 4.34 times higher than that in glycerol and glucose groups. The highest EGT productivity and the specific duty reached 0.85 mg/L/h and 8.40mg/g. When using the fructose as the carbon source, the cell dry weight was only 7.93 g/L but the EGT yield reached 22.69 mg/L with 2.86 mg/g, indicating that fructose was more favorable for EGT biosynthesis. When comparing the morphologies of different groups, we found molasses tended to form the small particles, while glycerol, glucose and fructose produced abundant mycelium in the broth, and maltose, sucrose and dextrin generated lots of large-size pellets. Combing with the results of EGT yield, we can conclude that small-size pellets might be more conducive to the synthesis of EGT.

In addition, it is well known that molasses contains about 50% carbohydrate, 10% of protein, a small amount of ash, trace amount of metal ions and vitamins, etc. Compared with other single component carbon sources, molasses could provide more micro-elements for EGT synthesis in *Panus conchatus*. In addition, molasses was one of the cheapest raw materials for the production of bioproducts (Koutinas, Wang, and Webb 2004), compared with glycerol and glucose, the price of molasses was 89.74 and 94.67% lower, implying that molasses was a more economic stock for EGT production.

3.4 Effect of oxygen supply conditions

Oxygen played important roles in cell growth and metabolic regulation of different natural product production. As shown in Fig. 3, cell growth was inhibited seriously at 60 rpm and the cell dry weight always kept below 1 g/L. However, when the rotation speed increased to 90rpm, the cell dry weight was

significantly increased, but the EGT yield remained the relative low level. Further improving the rotation speed to 120rpm and 150rpm, the cell growth stayed the same tendency and the highest cell dry weight reached 12 g/L but the EGT yield was significantly improved especially at 150 rpm. The highest EGT yield of 86.05mg/L was obtained at 150rpm after 144h fermentation, which was 113.69% higher than that of 120rpm.

In the EGT aerobic biosynthesis pathway, two enzymes of egt1 and egt2 were involved in the reactions. Oxygen and Fe^{2+} were used as cofactors in the catalytic reaction step of egt1. Therefore, higher oxygen supply was benefit for the synthesis of EGT. However, when the rotation speed continued to be increased, mycelium would grow on the bottle wall near the mouth of the shake flask, which was not conducive to the following cultivation. In addition, excessive oxygen supply would also increase intracellular ROS level, resulting in cytotoxicity (Quinzii et al. 2010). Hence, 150rpm was chosen for the further fermentation.

3.5 Adding precursors to enhance EGT biosynthesis

Histidine, cysteine and methionine are three key amino acids involving in the EGT biosynthesis, so appropriate amino acid supplementation should enhance the EGT production.

Histidine is the direct precursor of EGT providing the main backbone of the structure. As shown in Fig. 4, histidine addition did not affect cell growth obviously but could enhance the EGT production at the later fermentation stage. The EGT titer reached 135.52 mg/L when adding 0.2 g/L histidine, which was 54.28% higher than that of the control. However, increasing the histidine concentration could not improve the EGT production and 1 g/L histidine obviously decreased the EGT titer after 5 days fermentation, indicating that high concentration of histidine generated the obvious substrate inhibition.

Methionine could provide the methyl group during the EGT biosynthesis. As shown in Fig. 4c, lower concentration of methionine especially at 0.4g/L could promote the cell growth but methionine addition severely inhibited the EGT biosynthesis, indicating that the addition of exogenous methionine had a reversed effect on the synthesis of EGT in *Panus conchatus*. Different from our results, Lee et al. (Lee, Park, and Ahn 2009; Lee et al. 2009) found that the addition of methionine can significantly enhance the EGT production of *Ganoderma neo-japonicum* mycelia the same goes for *L. Edodes* mycelia proved by Tepwong et al. (Tepwong et al. 2012). which may be due to the differences in the absorption of different amino acids by different fungi. As for the cysteine addition, low concentration of cysteine could promote cell growth, but growth inhibition began to occur when the concentration increased to 0.8g/L. The highest cell dry weight reached 16.3g/L, which was 14.47% higher than that of the control. However, cysteine addition obviously enhanced the EGT production especially at the 6th day. The highest EGT titer of 148.79mg/L was obtained at 6th day when adding 0.4 g/L cysteine, which was 69.25% higher than that of control. The study of Lin et al. (Lin et al. 2015) also showed that cysteine was the most effective additive. Ergothioneine is a new sulfur-containing antioxidant similar to glutathione, which could provide sulfur source for biosynthesis by adding exogenous cysteine (Hidese, Mihara, and Esaki 2011).

3.6 Evaluation of antioxidant activity and stability of crude ergothioneine extract

To evaluate the antioxidant activity of EGT extract (EGTEX), two hydrosoluble antioxidants of vitamin C (VC) and glutathione (GSH) were used as the control. As shown in Fig. 5a, low concentration of VC and EGTEX obtained more than 90% of DPPH clearance rate while GSH showed poor DPPH scavenging ability at the same concentration. Although the DPPH scavenging ability of GSH was improved with the increase of the concentration, it was still lower than that of VC and EGTEX. Therefore, the crude extract of EGT processed the good antioxidant activity as VC and was better than that of GSH.

In order to further explore the stability of EGT produced by *Panus conchatus*, we studied the effect of temperature and pH on DPPH assay. As shown in Fig. 5c, the DPPH clearance rate of EGTEX was about 90% at both low and high temperature conditions, indicating that EGT has better thermal stability than vitamin C (Uprety and Revis 1964). The DPPH clearance rate was about 90% in the acidic condition even at pH 2-4 but dropped to about 80% at pH 5-6 and continued to drop to only 10% at pH 11. This indicated that EGT produced by the *Panus conchatus* was acid-resistant but not alkali-resistant, and was suitable for storage under acidic conditions. Unlike our results, Liu et al. (Liu et al. 2020) found that EGT from *Pleurotus citrinopileatus* was well tolerated in the range of pH 3-11. EGT extracted from different mushrooms might have different acid-base tolerances. In addition, we measured the pH of the fermentation liquid and the extract, and both of them were around 4.75. The fermentation environment of *Panus conchatus* was also acidic, which may be one of the reasons that the EGT produced by *Panus conchatus* was acid-resistant but not alkali-resistant.

4. Conclusion

In this study, the EGT production ability of a medicinal fungi of *Panus conchatus* was investigated from strain identification, process optimization to product evaluation. The highest EGT titer of 148.79mg/L was obtained and the crude EGT extract showed higher antioxidant activity with good stability. For the first time *Panus conchatus* was corelated with the EGT production, which could provide a new source for industrial production of EGT and new ideas for the exploration of *Panus conchatus*.

Declarations

Acknowledgements

Not applicable.

Data Availability

All data generated or analysed during this study are included in this published article.

Authors' contributions

Min Zhu and Yiwen Han designed the study. Lujing Ren and Xuechao Hu performed the assessment. Min Zhu and Lujing Ren wrote the manuscript. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

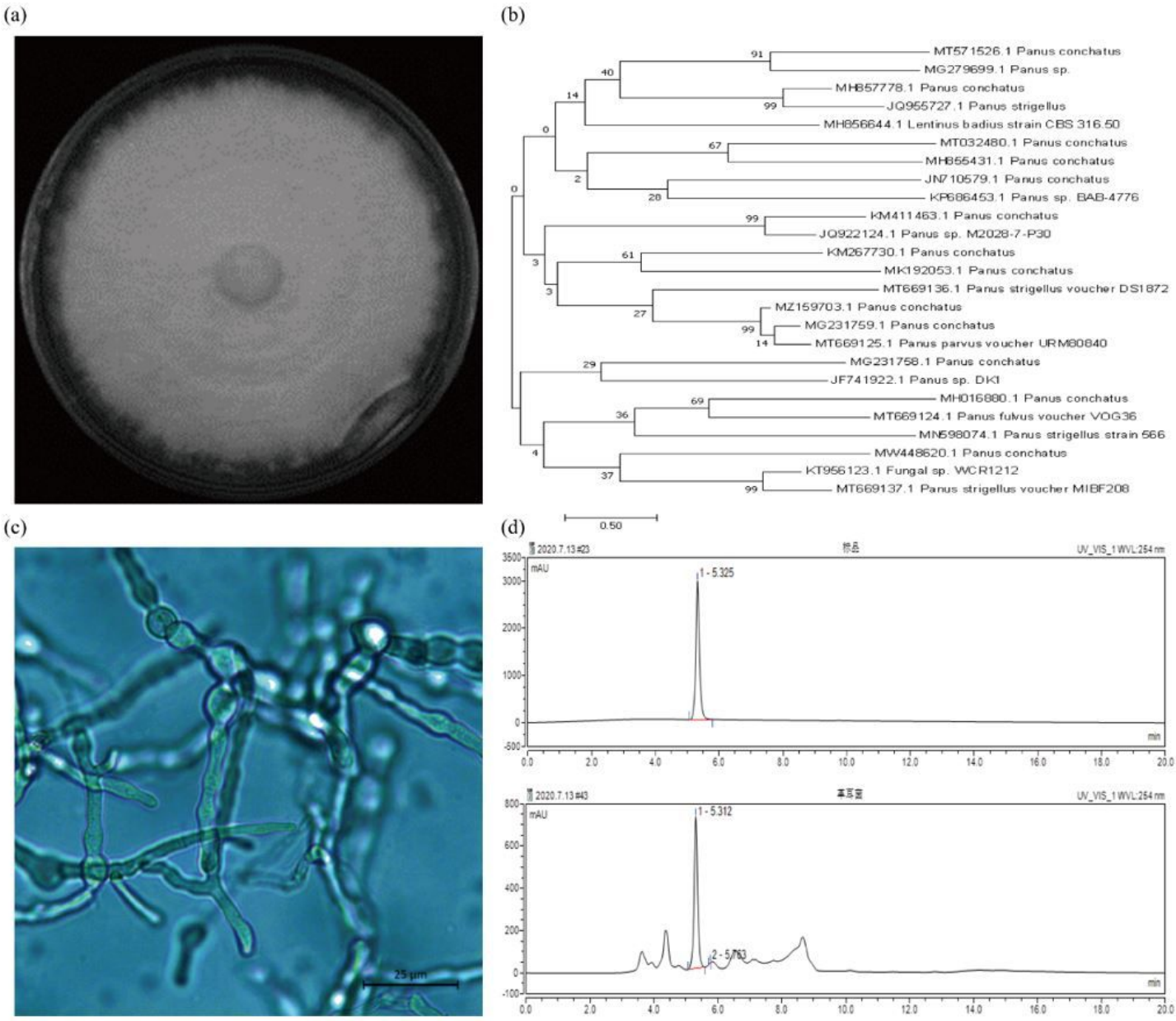

Figure 1

Fungal colony and product identification (a) Panus conchatus colony; (b) Phylogenetic tree of the Panus conchatus; (c) Panus conchatus mycelial morphology (a, LEICA DM1000, 40×magnification); (d) Liquid chromatogram for product determination (The top is the peak of the standard, and the bottom is the peak of the extract).

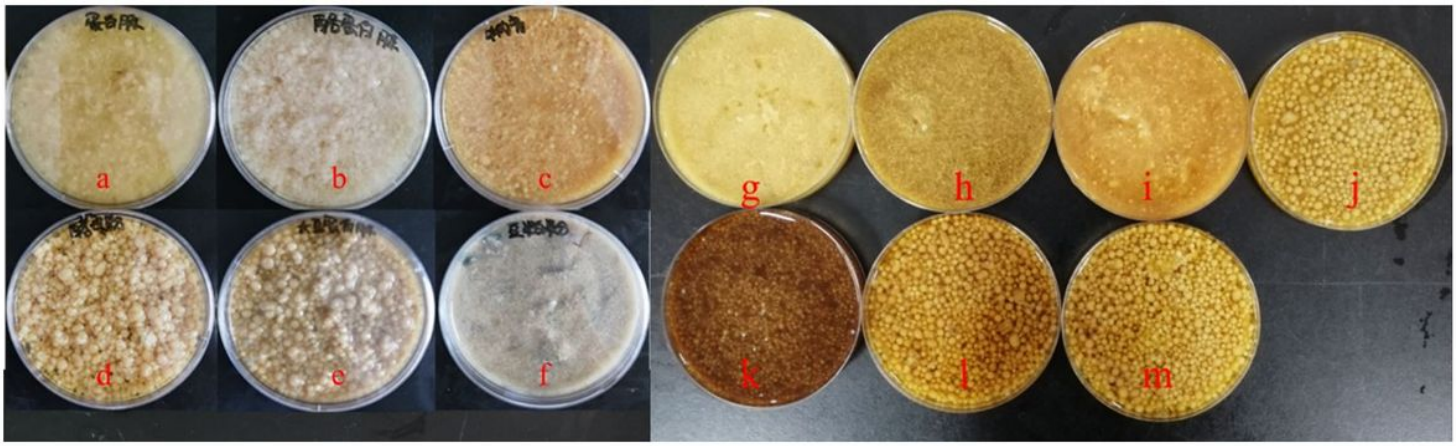

Figure 2

Effect of different medium on thallus morphology ((a) peptone, (b) casein peptone, (c) beef extract, (d) yeast powder, (e) soy peptone, (f) soybean meal powder, (g) glycerol, (h) glucose, (i) fructose, (j) dextrin, (k) molasses, (l) maltose, (m) sucrose).

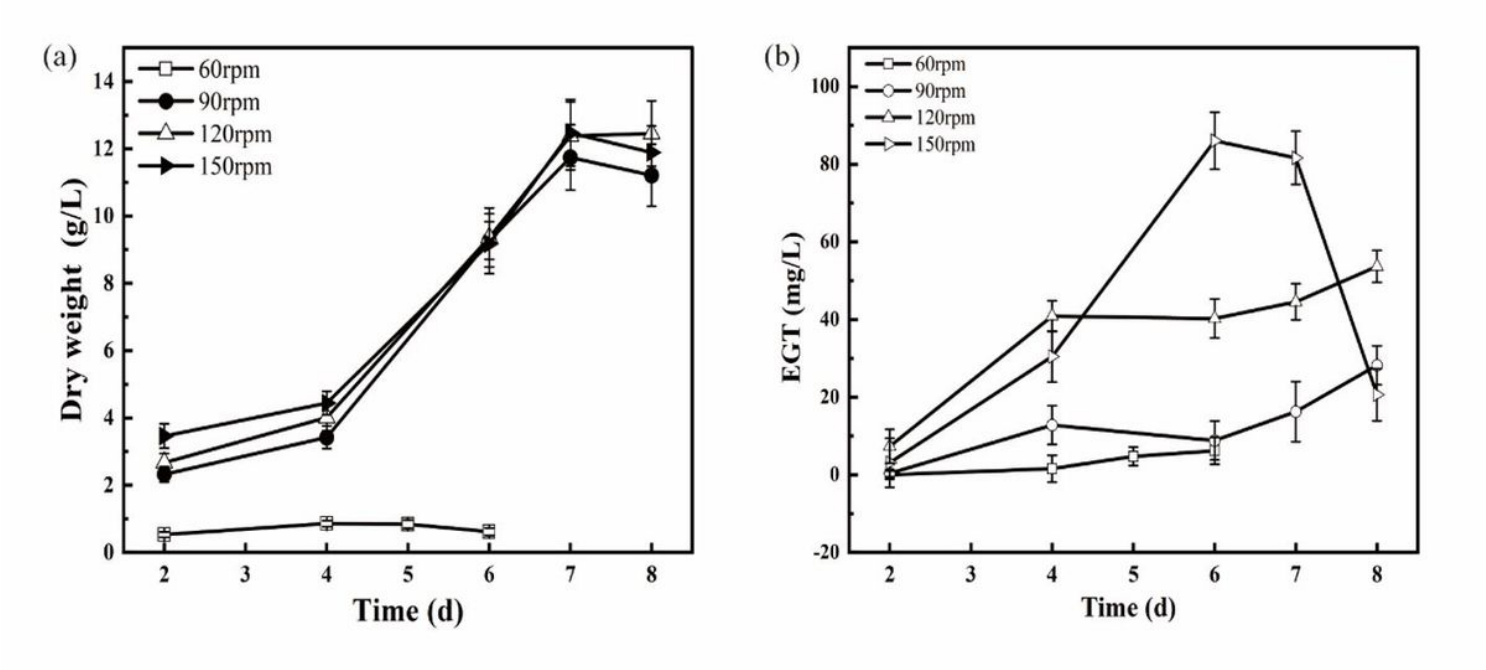

Figure 3

Effects of different rotations on dry weight and ergothioneine production of Panus conchatus. (a) dry weight (b) ergothioneine production.

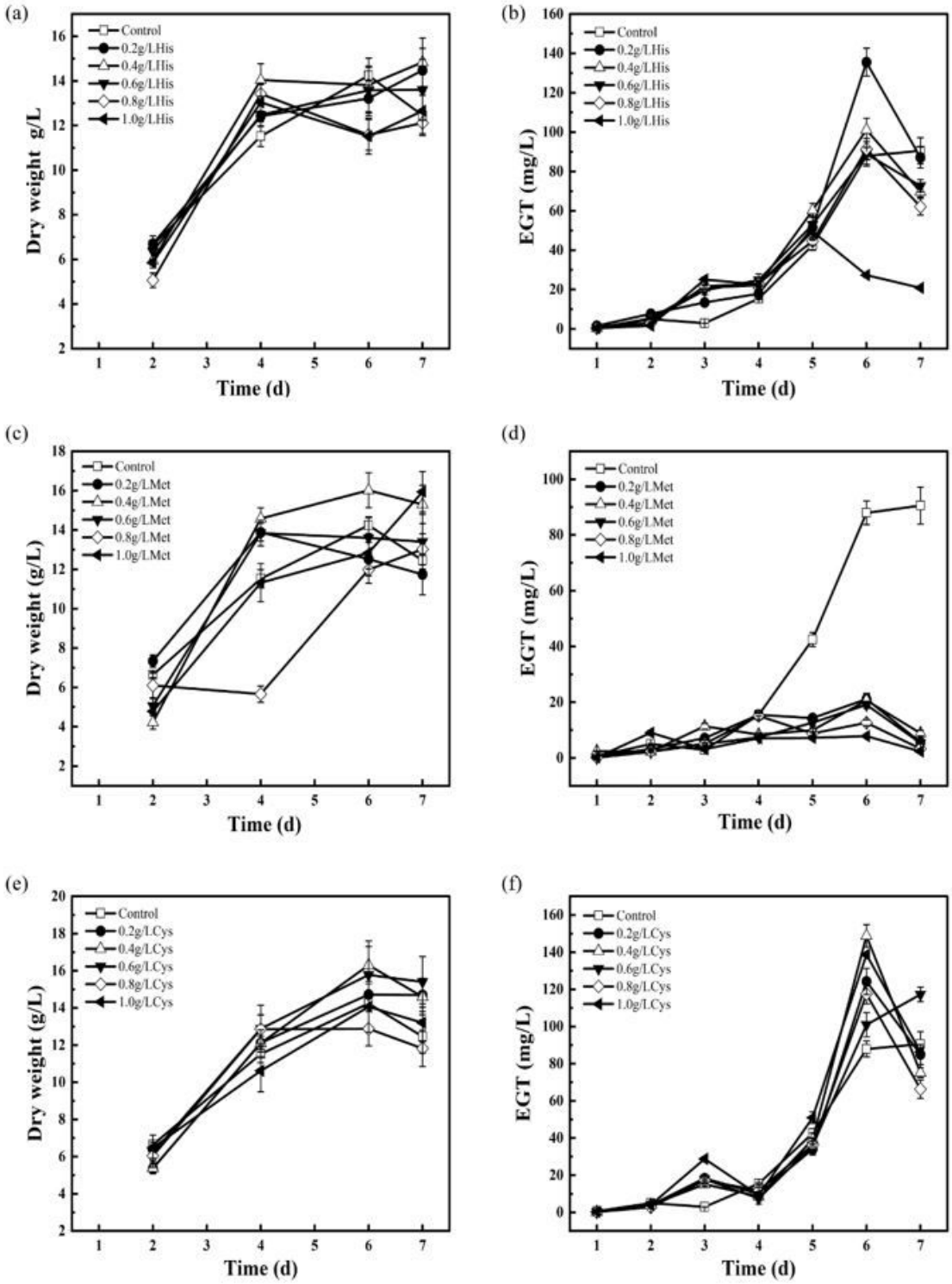

Figure 4

Effects of different concentrations of precursors on dry weight and ergothioneine production. (a, c, e) dry weight (b, d, f) ergothioneine production.

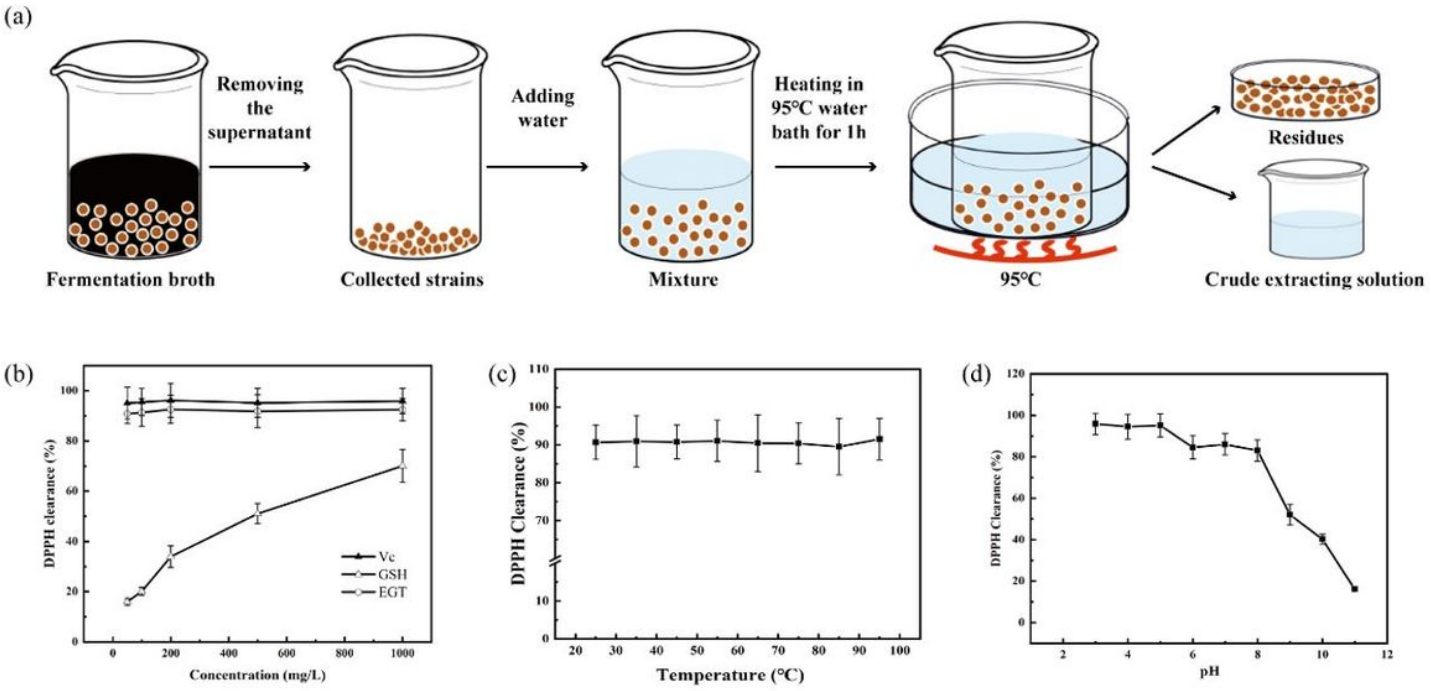

Figure 5

(a) Extraction of ergothioneine; (b) DPPH scavenging ability of ergothioneine, glutathione and vitamin C; (c) Effect of temperature on scavenging DPPH by ergothioneine from Panus conchatus; (d) Effect of pH on scavenging DPPH by ergothioneine from Panus conchatus.

Supplementary Files

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