Study on the Biological Materials Produced by Phellinus Igniarius Fermenting Spent Mushroom Substrate and Corn Cob

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Abstract. Submerged fermentation by *Phellinus igniarius* was done using solid wastes of spent mushroom substrate and corn cob in the medium. On the basis of single factor experiments, orthogonal test with four factors and three levels was carried out using contents of flavonoids, soluble protein and mycelia biomass in fermentation broth as main reference indexes. The results showed the optimal formula was 0.5% yeast extract, 0.2% KH_2PO_4, and 0.1% $MgSO_4$, when the ratio of wheat bran and the mix of spent mushroom substrate and corn cob (1:1) was 2:8, On the basis of optimum medium formula, three groups of parallel tests were carried out, the contents of flavonoids and soluble protein, mycelia biomass obtained were 0.465 mg/mL, 0.278 mu g/mL, and 1.378 g/100 mL respectively.

1. Introduction

The submerged fermentation of edible fungi not only shows the higher growth speed of mycelium and higher utilization rate of the nutrition, the mycelia obtained have a variety of nutrients and bioactive materials, but also the submerged ferments contain a wealth of primary metabolic products, such as proteins, polysaccharides and secondary metabolism products [1-5], such as flavonoids, antibiotics etc. The study will provide important references for developing the new kind of food and biological materials. Corn cob and spent mushroom substrate rich in nutritional substances was fired as agricultural wastes and thrown, which lead to waste of resource and environmental pollution. If it is collected, utilized and added in the fermentation culture medium of *Phellinus igniarius*, it will establish a new way of regenerating and applying [6-8]. In our study, *Phellinus igniarius* was used as material, the corn cob and spent mushroom substrate were added into the medium of liquid fermentation, contents of flavonoids and soluble protein and biomass in ferments were determined. The optimal medium formula was obtained by the orthogonal test and overall balance analysis which will provide references for *Phellinus igniarius* further development and open up a new way of application of spent mushroom substrate and corn cob.

2. Materials and Methods

2.1. Materials

Corn cob and wheat fran were bought from the market, spent mushroom substrate was provided by professor Ban. They were dried at 60°C, crushed and reserved over 60 mesh sieve. *Phellinus igniarius* strain maintained on potato agar and incubated at 28°C for 7 days was obtained from the biotechnology laboratory of Tianjin Agricultural College. Formula of liquid seed medium: 3% glucose, 0.3% peptone, 0.05%, $MgSO_4$, 0.1% KH_2PO_4, pH unadjusted.

2.2. Methods

2.2.1. Inoculation and Culture. 3~4 pieces of Phellinus igniarius with the size of 0.5 cm_2 were inoculated into liquid seed medium and cultured for 5 d at 25°C and 150 r/min. 10 mL of the first class seed solution was transferred to a new medium and continued to culture for another 5 d.

2.2.2. Determination of contents of flavonoid and soluble protein and mycelia biomass. The fermentation broth was centrifuged at 4000 rpm for 10 min, the supernatant was used to determine the contents of flavonoid and soluble protein. Detection of flavonoids was determined by $NaNO_2$-Al $(NO_3)_3$-NaOH colorimetry at 510 nm wavelength. With the concentration of lutin (mg/mL) as the horizontal coordinate, the absorbance as the vertical coordinate, the standard curve was drawn, the equation was y=0.3716x-0.0017, R^2=0.9976 with a good linear relationship. Detection of soluble protein was determined by the method of Coomassie brilliant blue G250. With protein concentration (mg/mL) as the horizontal coordinate, the absorbance as the vertical coordinate, and the standard curve was drawn at the 595 nm wavelength. The linear regression equation was obtained as follows: R^2=0.9971; y=5.8516x+0.0011, with a good linear relationship. The mycelium precipitate was washed three times by distilled water and dried at 80°C until constant weight, then weighed. Biomass of mycelium=weight of mycelium dried (g)/ volume of fermentation broth.

2.2.3. Experiments of single factor during the process of fermentation. In order to study the effect of different single factors on contents of flavonoids and soluble protein and biomass of mycelium in fermentation broth, experiments were designed like this: different incubation period, different ratio of wheat fran to the mix of spent mushroom substrate and corn cob, different inoculation amount, different pH of medium, different amount of yeast extract, different concentration of $MgSO_4$ and KH_2PO_4.

2.2.4. The orthogonal test. The orthogonal test of L9 (3^4) was designed with the ratio of bran and mix (A),concentrations of yeast extract powder (B), KH_2PO_4 (C) and $MgSO_4$ (D) as the factors. The factor level table was shown in Table 1.

Table 1. Orthogonal design table of four factors and three levels

Levels	A	B	C	D
1	2:8	0.4	0.05	0.10
2	3:7	0.5	0.10	0.15
3	4:6	0.6	0.15	0.20

Note: “mix”: the mix of spent mushroom substrate and corn cob.

3. Results and Conclusion

3.1. The effect of culture time on the Phellinus igniarius fermentation

Suitable culture time can not only maintain higher cell viability, but also achieve as much dry cell weight as [9]. According to fig.1, the contents of flavonoids and soluble protein during the process of

fermentation showed increased first and then decreased with the prolongation of the culture time. The contents of flavonoids and soluble protein reached to the highest of 0.395 mg/mL and 0.263 g/mL at the fourth day. According to fig.2, myceliua biomass increased first and then decreased, the maximum value was 1.051 g/100 mL. To sum up, the optimum culture time was the fourth day.

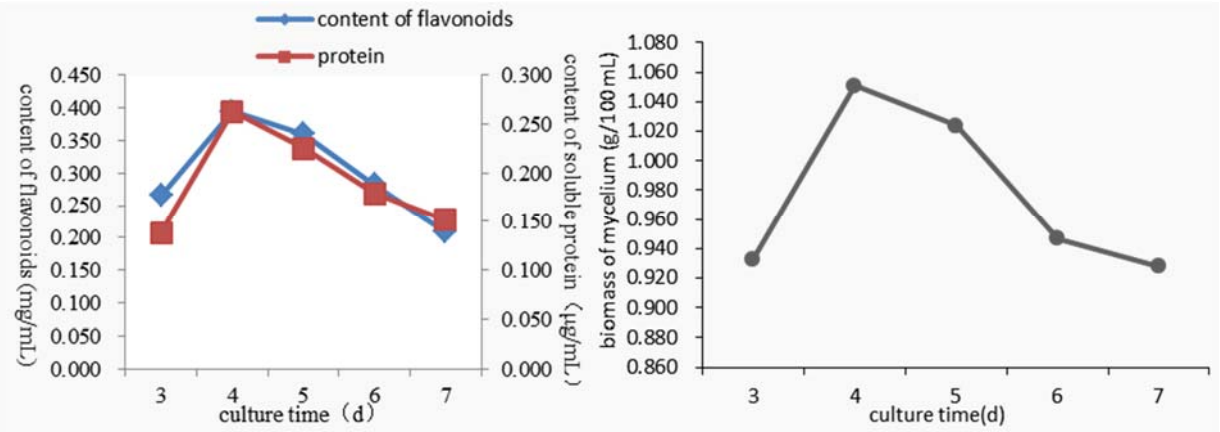

Figure 1. Effect of culture time on contents of flavonoids and soluble protein

Figure 2. Effect of culture time on mycelia biomass

3.2. The effect of the ratio of fran to mix on the Phellinus igniarius fermentation

Different concentrations of spent mushroom substrate and corncob added had a significant effect on contents of metabolites. Results were shown in figures 3 and 4. According to fig. 3, when the ratio of bran and mix was 3:7, the highest contents of flavonoids and soluble protein were 0.403 mg/mL and 0.289 g/mL respectively. From fig.4, when the ratio was 3:7, mycelia biomass had the highest value of 1.194 g/100 mL. In conclusion, the best ratio was 3:7.

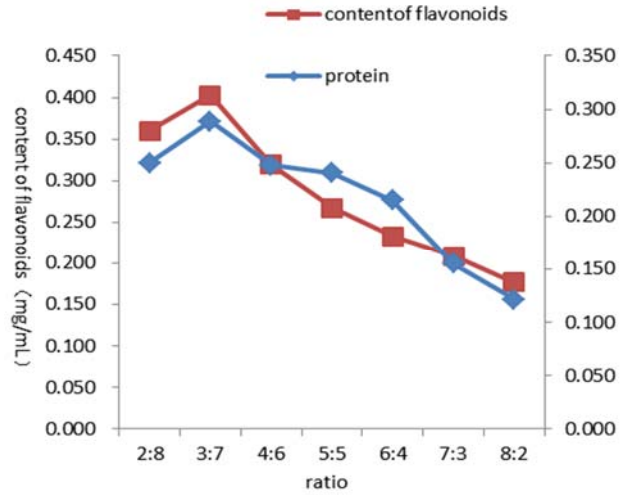

Figure 3. Effects of ratios on contents of flavonoids and soluble protein

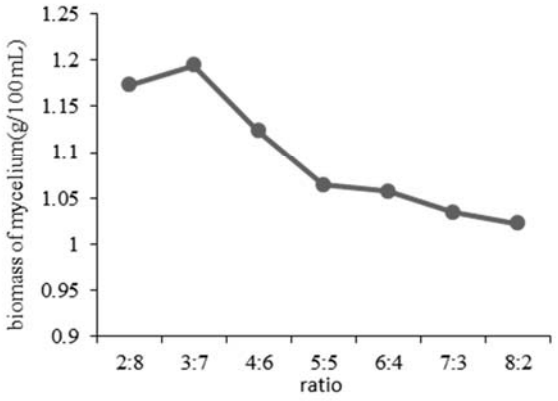

Figure 4. Effects of ratios on mycelia biomass

3.3. The effect of inoculation amount on the fermentation of Phellinus igniarius

In the process of fermentation, different inoculation amount affected metabolites of the fermentation broth. From fig.5 and 6, at 3% inoculation amount, contents of flavonoids and soluble protein reached

to a maximum of 0.387 mg/mLand 0.164 g/mL respectively, mycelia biomass was up to 1.185 g/100 mL. To sum up, the best amount of inoculation was at 3%.

3.4. The effect of pH on the fermentation of Phellinus igniarius

The Phellinus *igniarius* could grow in the medium of pH 3~7, but different pH value had a great influence on the contents of biological materials in fermentation liquid. Seen from fig.7, contents of Flavonoids and protein reached to the highest value of 0.395 mg/mL and 0.314 g/mL at pH 5. According to Fig.8, mycelia biomass showed the highest value of 0.923 g/100 mL at pH 5. To sum up, the best pH was 5.

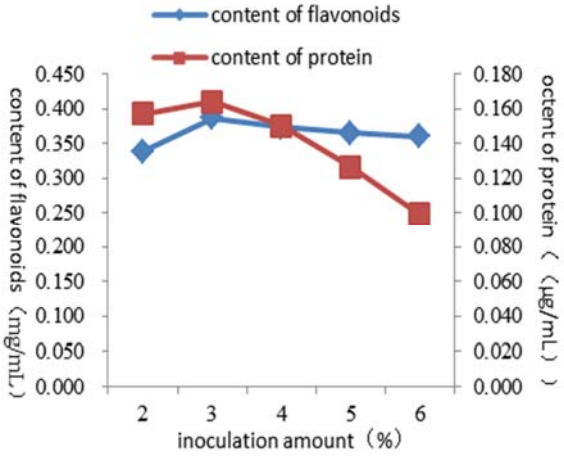

Figure 5. Effect of inoculation amount on contents of flavonoids and soluble protein

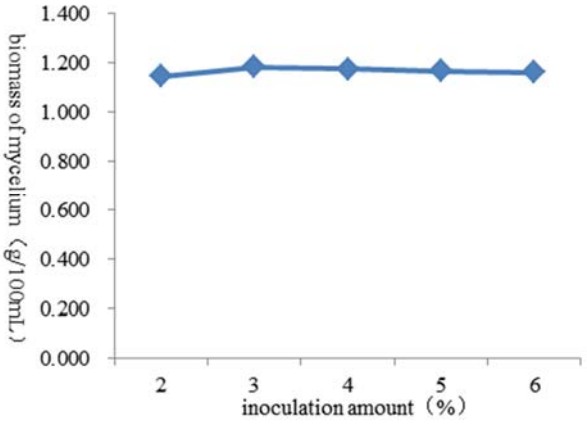

Figure 6. Effect of inoculation amount on mycelia biomass

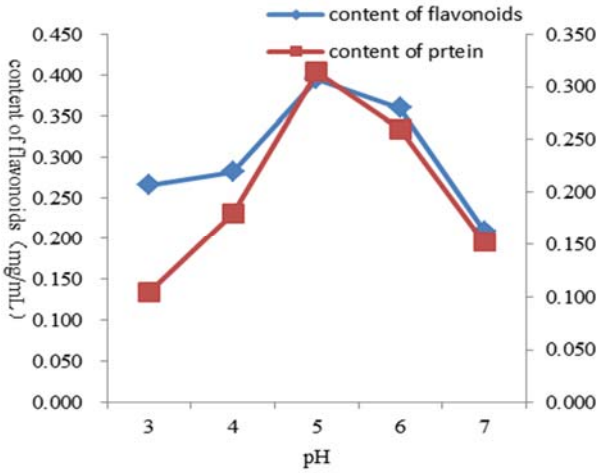

Figure 7. Effect of pH on contents of flavonoids and soluble protein

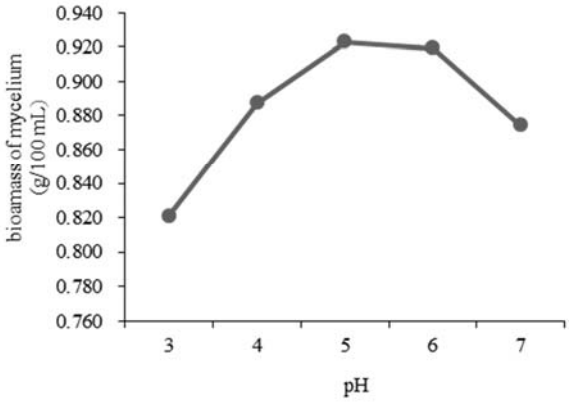

Figure 8. Effect of pH on mycelium biomass

3.5. Effect of yeast extract powder concentration on the fermentation of Phellinus igniarius

Nitrogen sources are essential for the formation of cell biomass and nitrogenous metabolites. Compound nitrogen sources are more conducive to mycelia growth than single bran nitrogen sources [14]. According to fig.9, the contents of flavonoids and soluble protein reached the highest value of 0.384 mg/mL and 0.232 g/mL respectively at 0.5%. From fig.10, mycelia biomass was up to the highest value of 1.212 g/100 mL at 0.6% yeast extract powder. To sum up, the best concentration was 0.5%.

3.6. The effect of MgSO4 concentration on the Phellinus igniarius fermentation

Inorganic salts (metal ions) played an important role in the growth of *Phellinus igniarius*. There were significant differences in effects of different kinds of inorganic salts on the growth of *Phellinus igniarius*. $MgSO_4$ might promote the synthesis of related enzymes of *Phellinus linteus* [13]. According to fig.11, contents of flavonoids and soluble protein were the highest when $MgSO_4$ content was 0.1%. According to fig.12, mycelia biomass reached the highest value of 0.819 g/100 mL at 0.15% $MgSO_4$. To sum up, the content of $MgSO_4$ was 0.1%.

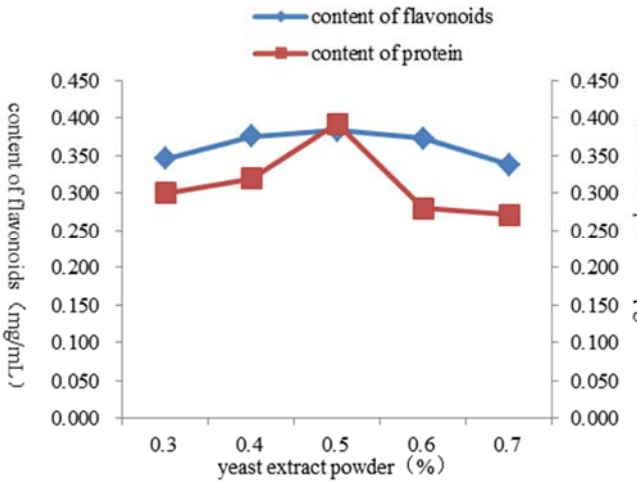

Figure 9. Concentrations of yeast extract powder contents of flavonoids and soluble protein

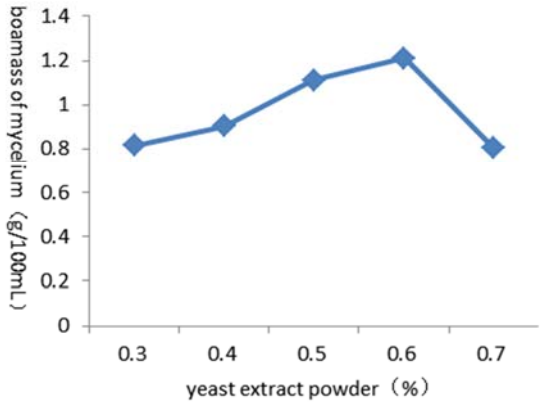

Figure 10. Effect of yeast extract content on mycelia biomass

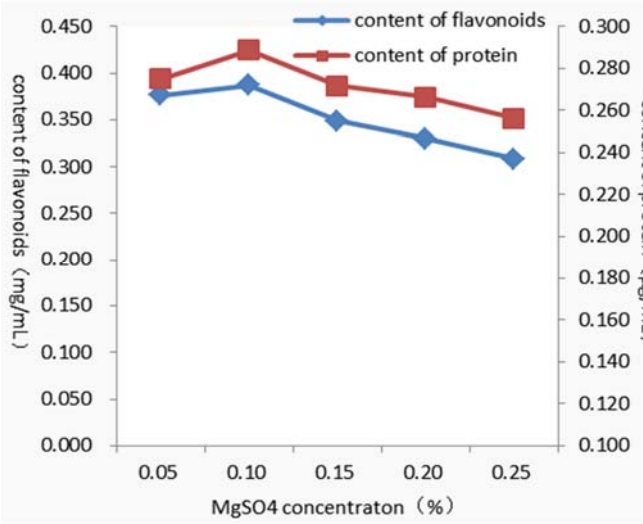

Figure 11. Effect of the concentration of $MgSO_4$ flavonoid and soluble protein content

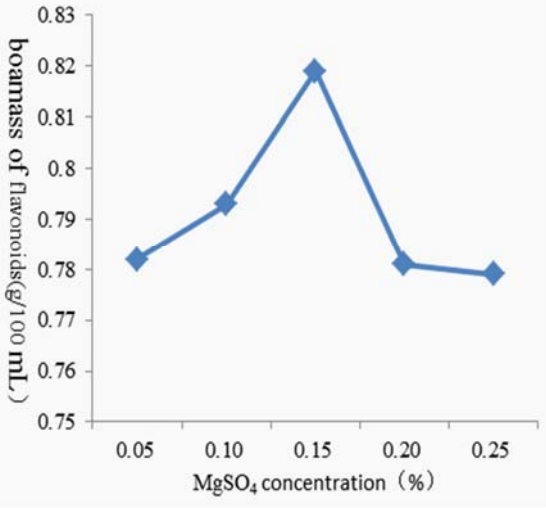

Figure 12. Effect of the concentration of on $MgSO_4$ on mycelia biomass

3.7. The effect of KH2PO4 concentration on the fermentation of Phellinus igniarius

KH_2PO_4, as an inorganic element, is an indispensable element in the growth of microbes. It can provide phosphorus elements for the synthesis of genetic material and K elements for the normal physiological function of bacteria. It is a component of the cells and enzymes that effectively regulate the osmotic pressure and pH of cell [15]. According to fig.13, contents fo flavonoids and soluble protein had the highest values of 0.446 mg/mLand 0.384 g/mL at 0.15% KH_2PO_4. According to fig 14, mycelia biomass

was up to the highest value of 1.168 g/100 mL at 0.15% KH_2PO_4. To sum up, the content of KH_2PO_4 was 0.15%.

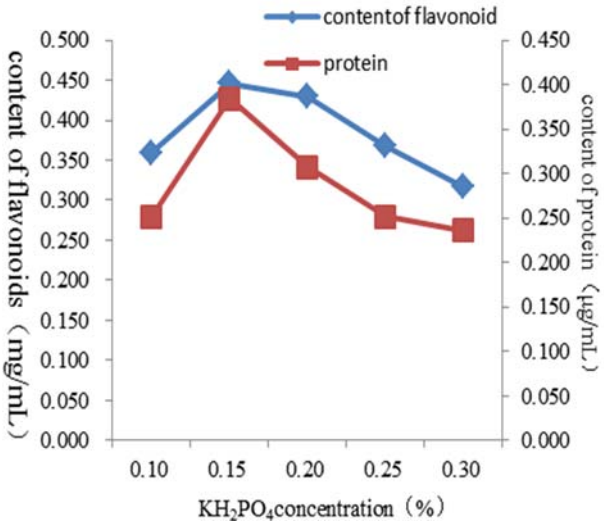

Figure 13. Effect of KH_2PO_4 concentration on of flavonoids and soluble protein

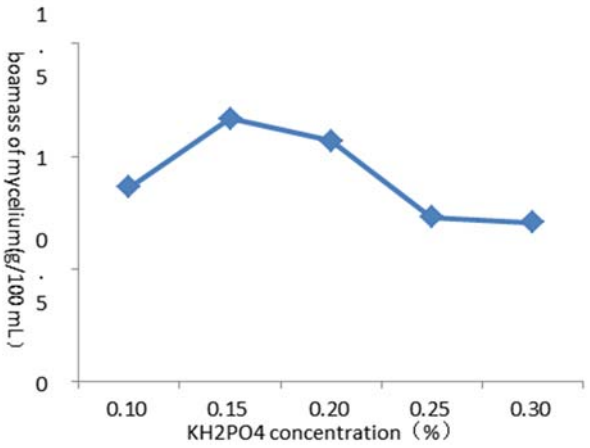

Figure 14. Effect of KH_2PO_4 concentration contents on mycelia biomass

3.8. Orthogonal test result

The results of the orthogonal test were shown in Table 2.

Table 2. Orthogonal Test Result

Test No.	A	B	C	D	Flavonoids (mg/mL)	Protein (μg/mL)	Biomass (g/100 mL)
1	1	1	1	1	0.384	0.277	1.033
2	1	2	2	2	0.379	0.227	1.044
3	1	3	3	3	0.387	0.350	1.109
4	2	1	2	3	0.416	0.174	1.570
5	2	2	3	1	0.384	0.118	1.400
6	2	3	1	2	0.311	0.193	1.219
7	3	1	3	2	0.293	0.087	1.064
8	3	2	1	3	0.365	0.201	1.457
9	3	3	2	1	0.284	0.109	1.441
Kh_1	0.383	0.364	0.353	0.351			
Kh_2	0.370	0.376	0.360	0.328			
Kh_3	0.314	0.327	0.355	0.389			
R	0.069	0.049	0.007	0.061			
Kd_1	0.285	0.179	0.224	0.168			
Kd_2	0.162	0.182	0.170	0.169			
Kd_3	0.132	0.217	0.185	0.242			
R	0.153	0.038	0.054	0.074			
Kj_1	1.262	1.422	1.436	1.491			
Kj_2	1.596	1.500	1.552	1.309			
Kj_3	1.521	1.456	1.391	1.579			
R	0.334	0.078	0.131	0.270			

According to table 2, the best medium formula for flavonoid content was $A_1B_2C_2D_3$ but for soluble protein was $A_1B_3C_1D_3$, and for mycelium biomass was $A_2B_2C_2D_3$. The medium formula for three indexes was not the same. In order to take an account of all three indicators, mult-iindex orthogonal test analysis was adopted. The effects of Factor A on each index: the ranges of the contents of flavonoids and soluble protein were both greater, indicating that the factor A was the most influential factor, A_1 level was the best in terms of flavonoid content and soluble protein content, and A_2 level was best for mycelia biomass, three indicators were integrated, and A_1 level was selected; The influence of factors B on each index: B_2 level was best for flavonoids and mycelia biomass, but B_3 level was the best for soluble protein content, three indicators were integrated, B_2 level was selected; The influence of C on each index: the range of flavonoid content was relatively small, soluble protein content was much greater, and C1 level was the best, the result indicated that $MgSO_4$ was a major factor affecting protein content, in term of flavonoid content, C_2 level was the best and B_2 level was best for mycelial biomass, to sum up, it was taken C_2 level for $MgSO_4$;The influence of D on each index: D_3 was the best for all of three indexes of flavonoids content, soluble protein content and mycelia biomass. Through the above comprehensive analysis, it was concluded that the best medium formula for three indexes was $A_1B_2C_2D_3$: bran and mix (1:1) 2:8, 0.5% yeast extract powder, 0.2% KH_2PO_4, 0.1% $MgSO_4$.

3.9. Verification test

Phellinus igniarius had been cultured for 4 d under the condition of inoculum amount of 3%, pH5, at 25 C, and 150 r/min in order to do the parallel validation test. Contents of flavonoids and soluble protein in the fermentation broth were 0.455 mg/mL and 0.271 g/mL, and mycelia biomass was 1.378 g/100 mL. Compared with the reported, they all had been promoted [13-15].

4. Discussion

In recent years, the industry of mushroom has developed rapidly, and the amount of solid waste, namely the spent mushroom substrates has been increasing. According to the survey, about 3.25 kg solid wastes were harvested when getting 1 kg mushroom. People usually discarded the solid waste after harvesting [16]. The corn cob was usually used as fuel, or directly discarded, and a great deal of incineration of corn core will cause environmental pollution. This experiment studied the use of solid waste spent mushroom substrate and corn cob as medium for submerged fermentation of *Phellinus igniarius*. Mushroom substrate and corn cob are easier to obtain and 5 times cheaper than the corn flour and soybean powder used in other literatures, in the meantime, the content of flavonoids and the biomass of mycelium were promoted comparatively [14].

5. Conclusion

Through multi-index orthogonal test, the best formula of medium was obtained: the concentration ratio of bran with mix (1:1) was 2:8, 0.5% yeast extract, 0.20% KH_2PO_4 and 0.10% $MgSO_4$. Under these conditions, the flavonoid content was 0.455 mg/mL, the protein content was 0.271 g/mL, and the mycelium biomass was 1.378 g/100 mL.

Acknowledgments

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