

Research of Submerge Fermentation Conditions on *Agaricus blazei* Murill

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Abstract

Rotational speed, temperature, inoculation amount and pH are the four factors that affect the submerge fermentation of *Agaricus blazei* Murill. The orthogonal experiment was on the basis of single factor experiments. And the optimal submerge fermentation condition of *Agaricus blazei* Murill was determined through orthogonal test. The results were as follows: temperature (25°C), rotational speed (150 r/min), inoculation amount (6%) and pH (7), in this condition the cell dry weight reached 1.32 g/100mL; temperature (25°C), rotational speed (150 r/min), inoculation amount (8%) and pH (6.5) in this condition the extracellular polysaccharide reached 6.95 mg/mL.

Keywords

Agaricus blazei Murill, Submerged Fermentation, Cell Dry Weight, Extracellular Polysaccharide

1. Introduction

Agaricus blazei Murill was considered one of the most important edible and medicinal mushrooms [1]. We also called it *Agaricus brasiliensis*. According to the research, *Agaricus blazei* Murill has certain medicinal function and health care function [2], such as immune-enhancement, oxidation resistance, lower blood lipids *et al.* [3]. So the study on *Agaricus blazei* Murill shows good application future [4]. Now it has realized the artificial cultivation of *Agaricus blazei* Murill but it was still difficult to cultivate it, while the limitations of time and yield cannot meet the market demand. Research showed that there were no essential difference between fruiting bodies and mycelium of *Agaricus blazei* Murill in the composition of polysaccharide [5]. Besides liquid fermentation has much more advance than solid fermentation. Firstly, products have a steady good quality and yield well; secondly, the fermentation conditions are easy to control; thirdly, the cycle of the liquid fermentation is shorter than solid fermentation [6].

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The experiment was to study the effects on different conditions submerge fermentation of *Agaricus blazei* Murill, which was based on the optimization of submerged cultivation with biomass and production of bacterial extracellular polysaccharides as experimental indicators. The optimal submerge fermentation conditions of *Agaricus blazei* Murill were obtained through the experiment.

2. Materials and Methods

2.1. Materials

The *Agaricus blazei* Murill strains were brought from Culture Collection Center of Huazhong Agricultural University.

2.2. Medium

Solid culture medium

Potato 200 g, Glucose 20 g, KH_2PO_4 2 g, $\text{MgSO}_4 \cdot 5\text{H}_2\text{O}$ 0.2 g, Agar 20 g, H_2O 1000 mL, pH 6.5 - 7.5

Liquid seed medium

Potato 200 g, Glucose 20 g, KH_2PO_4 2 g, $\text{MgSO}_4 \cdot 5\text{H}_2\text{O}$ 0.2 g, H_2O 1000 mL, pH 6.5 - 7.5

Liquid fermentation medium

Potato 200 g, Bran 50 g, Glucose 20 g, KH_2PO_4 2 g, $\text{MgSO}_4 \cdot 5\text{H}_2\text{O}$ 0.2 g, H_2O 1000 mL, pH 6.5 - 7.5

Sugar 57.5 g/L, Industrial corn pulp 2.05 g/L, KH_2PO_4 2 g, $\text{MgSO}_4 \cdot 5\text{H}_2\text{O}$ 0.2 g, H_2O 1000 mL

2.3. Experimental Method

At the end of fermentation, centrifuge the fermentation solution under 2500 r/min. Distilled water was used to wash the mycelium for 4 - 5 times, then dried the mycelium pellet in the air drier at 60°C. DNS method [7] [8], Phenol-Sulfuric acid method [9]-[11], Roe colorimetric method [12] was used to determine sugar, total sugar and sucrose. The content of the extracellular polysaccharide is equal to total sugar minus sucrose and reducing sugar.

2.4. *Agaricus blazei* Murill Liquid Fermentation Process

The shaker was used during the process of the liquid fermentation of *Agaricus blazei* Murill. pH, temperature, rotational speed, inoculation amount are the four factors that affect the submerge fermentation of *Agaricus blazei* Murill. During the liquid fermentation of *Agaricus blazei* Murill, we should control the four factors. This ensured the optimum fermentation conditions. The dry weight of biomass and extracellular polysaccharide were taken as the detection index.

2.5. Single Factor Experiment

Under the aseptic conditions, seed solution of *Agaricus blazei* Murill was inoculated in 200 ml flasks which included 100 ml liquid fermentation medium, the inoculum size was 5% and the pH of the medium respectively were 5.5, 6.0, 6.5, 7.0 and 8. Then the flasks were cultured 3 days in constant temperature oscillation shaker, the culture temperature was 25°C and the rotating speed was 150 r/min.

Under the aseptic conditions, seed solution of *Agaricus blazei* Murill was inoculated in 200 ml flasks which included 100 ml liquid fermentation medium, the inoculum size was 5%. Then the flasks were cultured 3 days in constant temperature oscillation shaker, the culture temperature was 25°C and the rotating speed respectively were 90 r/min, 120 r/min, 150 r/min, 180 r/min and 210 r/min.

During the single factor experiment of inoculum size, the 5 experiment gradient were adopted in experiment, which were 4%, 6%, 8%, 10% and 12%. Under the aseptic conditions, seed solution of *Agaricus blazei* Murill was inoculated in 200 ml flasks which included 100 ml liquid fermentation medium, the inoculum size respectively were 4%, 6%, 8%, 10% and 12%. Then the flasks were cultured 3 days in constant temperature oscillation shaker, the culture temperature was 25°C and the rotating speed was 150 r/min. The experiment indexes were cell dry weight and extracellular polysaccharide content.

During the single factor experiment of culture temperature, the 5 experiment gradient was adopted to determine optimal temperature in experiment, which were 19°C, 22°C, 25°C, 28°C and 31°C. Under the aseptic con-

ditions, seed solution of *Agaricus blazei* Murill was inoculated in 200 ml flasks which included 100 mL liquid fermentation medium, the inoculum size was 5%. Then the flasks were cultured 3 days in constant temperature oscillation shaker, the rotating speed was 25°C and the culture temperature respectively were 19°C, 22°C, 25°C, 28°C and 31°C. The experiment indexes were cell dry weight and extracellular polysaccharide content.

When performing temperature experiment, four gradients of 19°C, 22°C, 28°C, 31°C were set in single factor experiments to determine the optimum temperature of liquid fermentation of *Agaricus blazei*, the detection index is the same as the above. Under sterile condition, 5% of inoculum concentration is inoculated to a triangle bottle of 100 ml of liquid fermentation medium, and then cultured at the speed of 150 r/min, the temperature of 19°C, 22°C, 28°C, 31°C in constant temperature oscillation 3d respectively, measuring its extracellular polysaccharide contents and dry weight of biomass.

3. Analysis of Results

3.1. The Influence of pH on the Liquid Fermentation of *Agaricus blazei* Murill

Figure 1 which based on the residual sugar contents and extracellular polysaccharide content is used to examine the influence of different pH on the liquid fermentation of *Agaricus blazei*, while **Figure 2** which based on the dry weight biomass per 100 ml bacteria liquid bacteria is taken to identify the effect of different pH on *Agaricus blazei*. **Figure 1** illustrates that the relationship between metabolite accumulations and the growth of *Agaricus blazei* is similar to positive correlation. When pH ranges from 5.5 to 6.5, dry weight biomass and extracellular polysaccharide contents increased; if pH is more than 6.5, it decreased, **Figure 2** shows that *Agaricus blazei* can grows relatively well between pH 6 and 7 and grows best at pH 6.5, and its extracellular polysaccharide contents and dry weight biomass reach 3.85 mg/mL, 1.118 g/100mL respectively.

3.2. The Influence of Rotational Speed on Liquid Fermentation of *Agaricus blazei* Murrill

Ditto detection index, impact speed of *Agaricus* liquid fermentation can increase the amount of dissolved oxygen in the medium, this provides dissolved oxygen required of *Agaricus* normal growth and metabolism; on the other hand, speed can provide some centrifugal shear force that will cut down division sites of mycelium fungus

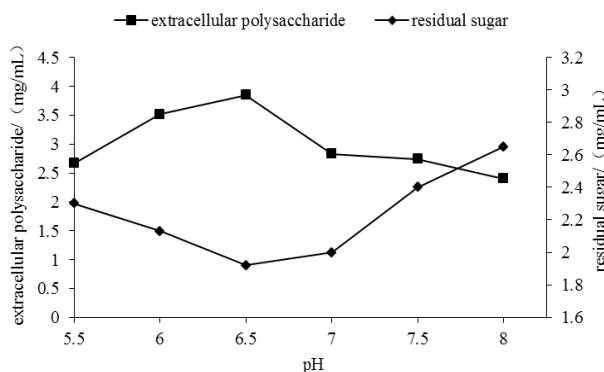


Figure 1. The effects of different pH on the submerge fermentation of *Agaricus blazei* Murill.

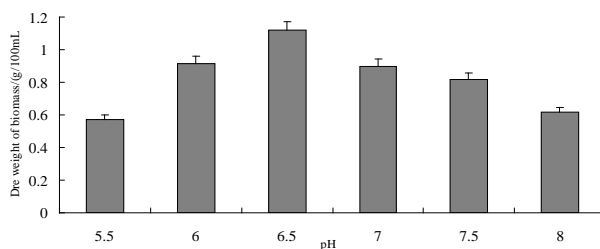


Figure 2. The effects of different pH on the submerge fermentation of *Agaricus blazei* Murill.

ball surface to form smaller ball in order to avoid the formation of excessive bacteria.

From **Figure 3**, **Figure 4** can be seen in 90 - 150 r/min either extracellular polysaccharide content or mycelium dry weight increases with increasing speed, at the same the amount of residual sugar have a downward trend, but when the speed exceeds 150 r/min showing a downward trend with increasing speed, then the amount of residual sugar begins to rise. To determine the optimum fermentation speed 150 r/min from experiments, extracellular polysaccharide content and cell dry weight, respectively 4.949 mg/mL, 1.078 g/100mL.

3.3. Effects of Inoculation on Liquid Fermentation of *Agaricus blazei* Murrill

Inoculum is directly related factors of *Agaricus* liquid fermentation, as the liquid parent species when inoculated using bacteria grow well in the growth stage of fermentation, so when inoculated into the fermentation medium can quickly adapt to the new culture environment, cell have a rapid value-added.

Figure 5 and **Figure 6** can be seen that when the inoculum is 4%, whether extracellular polysaccharide or cell

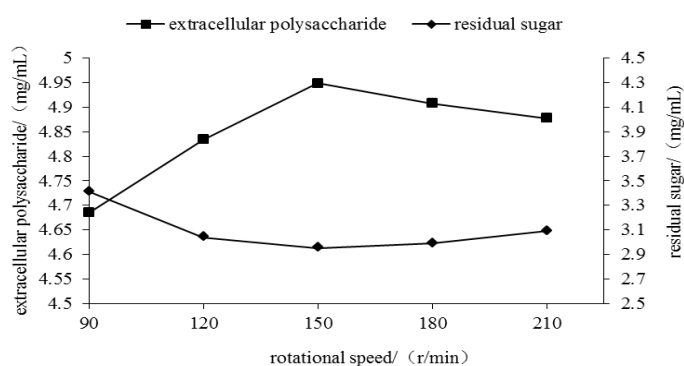


Figure 3. The effects of different rotational speed on the submerge fermentation of *Agaricus blazei* Murill.

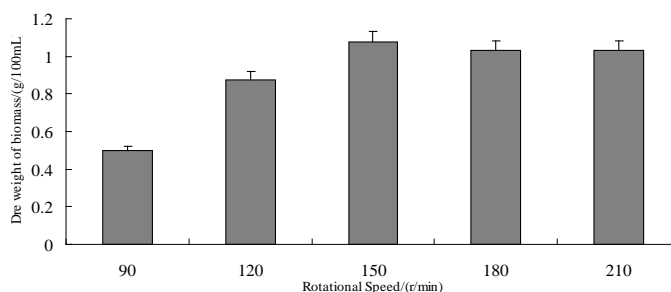


Figure 4. The effects of different rotational speed on the submerge fermentation of *Agaricus blazei* Murill.

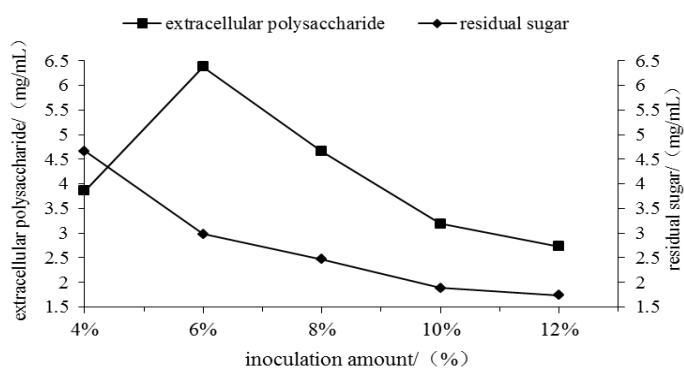


Figure 5. The effects of different inoculation amount on the submerge fermentation of *Agaricus blazei* Murill.

dry weight values is minimal, this may be due to the small base of the inoculated cells, the reason of the slow appreciation of the cell number. When the amount of inoculum is 8%, 10% and 12%, extracellular polysaccharide content and dry cell weight has increased to some extent compared with 4% inoculum, but smaller than when inoculum is 6%, this is probably because when the inoculum amount exceeds 6%, the cell base is large, cell values rapidly, this speeds up the consumption of nutrient medium and medium oxygen consumption, sufficient to provide nutrients and oxygen supply needed for normal growth of bacteria. From the experiments we can determine the amount of 6% is the optimum inoculation inoculum, then extracellular polysaccharide content and cell dry weight, respectively 6.38 mg/mL, 1.114 g/100mL.

3.4. Effect of Temperature on Liquid Fermentation of *Agaricus blazei* Murrill

The impact of temperature on the value of the cell *Agaricus* is one important factor, because any biochemical enzymatic reactions are associated with temperature changes. Effect of temperature on the fermentation is multifaceted and complex, mainly in the cell growth and product synthesis, physical properties of the fermentation broth and biosynthetic aspects.

From **Figure 7**, **Figure 8**, it can be seen, with the rise in temperature of 25°C before either dry cell weight or extracellular polysaccharide concentrations are elevated along, but when the temperature is above 25°C, showing a downward trend, may be due to a rise in temperature causing bacterial enzyme activity related to cell growth and metabolism reduce, so easy to make bacteria premature age, showing the results presented in the figure. Through experiments we can infer that 25°C is the optimum growth temperature of liquid fermentation, then extracellular polysaccharide content and cell dry weight respectively 4.408 mg/mL, 1.136 mg/mL.

3.5. Orthogonal Optimization Test of *Agaricus* Liquid Fermentation Culture Conditions

Based on the above results of single factor, doing orthogonal test of four factors and three levels about pH, speed, inoculum, temperature of affecting *Agaricus* liquid fermentation, factor level is as shown in **Table 1**, each level

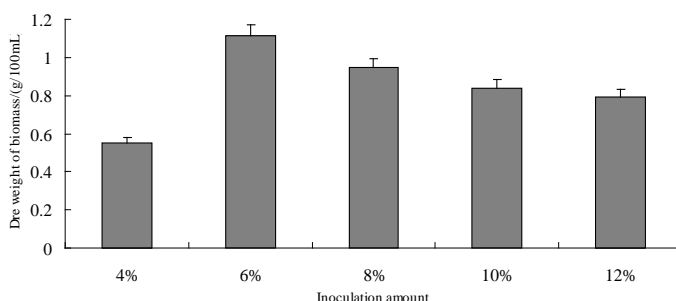


Figure 6. The effects of different inoculation amount on the submerge fermentation of *Agaricus blazei* Murill.

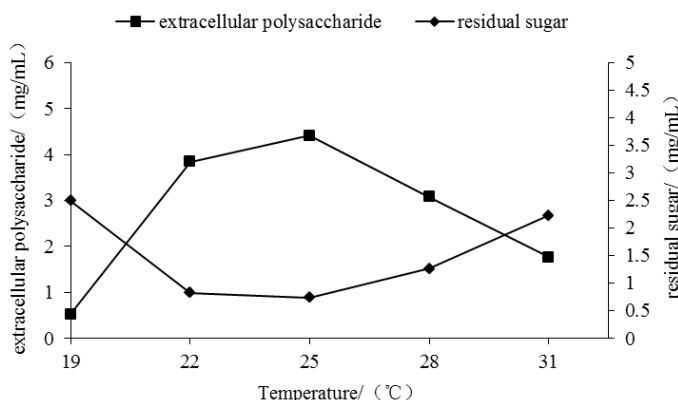


Figure 7. The effects of different temperature on the submerge fermentation of *Agaricus blazei* Murill.

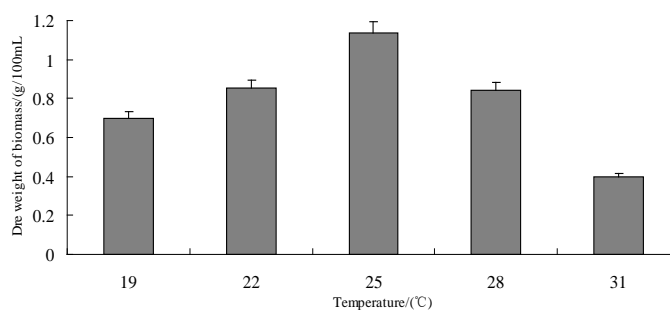


Figure 8. The effects of different temperature on the submerge fermentation of *Agaricus blazei* Murill.

Table 1. Orthogonal factors table.

Level	A Temperature (°C)	B Rotational speed (r/min)	C pH	D Inoculation amount (%)
1	22	120	6.0	4
2	25	150	6.5	6
3	28	180	7.0	8

of the test would be repeated three times or more.

3.6. Orthogonal Experimental Results with Dry Weight of Biomass as Detection Index

Range R indicates the level of impact factors on the experimental results, the size of range R represents the level of impact factors on the experimental results. From **Table 2**, according to the range R can be obtained four factors effect on the cell dry weight: Temperature > Rotational speed > Inoculation amount > pH, analysis of variance was used to test the various factors that impact on the experiment significantly, we can see from **Table 3**, temperature and speed have great effect on the *Agaricus blazei* Murrill fermentation liquor, the inoculation has amount effect on *Agaricus blazei* Murrill fermentation liquor significantly, however, pH has little influence on the fermentation liquor. According to the size of K value from table, we can draw the preferred combination of culture conditions: A₂B₂C₃D₂. This is inconsistent with the highest cell dry weight experiment experimental combination A₂B₂C₃D₁, hence the need for verification experiment. By experimental verification fermentation obtained by a combination of A₂B₂C₃D₂ cell dry weight 1.32 g/100ml, so A₂B₂C₃D₂ is the optimal experimental combination, that: temperature (25°C), speed (150 r/min), pH (7), inoculation amount (6%) for the optimal experiment combined to produce biomass as the goal, the dry cell weight can reach 1.32 g/100mL.

3.7. Orthogonal Experimental Results with Extracellular Polysaccharide as Detection Index

Range R* indicates the level of impact factors on the experimental results, the size of range R* represents the level of impact factors on the experimental results. From **Table 2**, according to the range R* can be obtained four factors effect on the extracellular polysaccharide: Temperature > Rotational speed > pH > Inoculation amount, analysis of variance was used to test the various factors that impact on the experiment significantly, we can see from **Table 4**, temperature and speed have great effect on the *Agaricus blazei* Murrill fermentation liquor, the inoculation has amount effect on *Agaricus blazei* Murrill fermentation liquor significantly, however, pH has little influence on the fermentation liquor. According to the size of K* value from table, we can draw the preferred combination of culture conditions: A₂B₂C₂D₃. This is inconsistent with the highest extracellular polysaccharide experiment experimental combination A₂B₂C₂D₃, hence the need for verification experiment. By experimental verification fermentation obtained by a combination of A₂B₂C₂D₃ cell dry weight 6.95 mg/mL, so A₂B₂C₂D₃ is the optimal experimental combination, that: temperature (25°C), speed (150 r/min), pH (6.5), inoculation amount (8%) for the optimal experiment combined to produce biomass as the goal, the dry cell weight can reach 6.95 mg/mL.

Table 2. Orthogonal test results and range analysis.

NO	Factor				Dry weight of biomass (g/100mL)	Extracellular polysaccharide/(mg/mL)
	A Temperature (°C)	B Rotational speed (r/min)	C pH	D Inoculation amount (%)		
1	22	120	6.0	4	0.783	5.12
2	22	150	6.5	6	1.073	6.15
3	22	180	7.0	8	0.731	5.05
4	25	120	6.5	8	0.988	6.85
5	25	150	7.0	4	1.318	6.83
6	25	180	6.0	6	1.114	6.78
7	28	120	7.0	6	0.874	4.15
8	28	150	6.0	8	0.991	5.36
9	28	180	6.5	4	0.806	4.95
K1	2.497	2.655	2.888	2.907		
K2	3.420	3.382	2.867	3.061		
K3	2.670	2.651	2.923	2.710		
R	0.278	0.245	0.018	0.117		
K1*	16.32	16.12	17.26	16.90		
K2*	20.46	18.34	17.95	17.08		
K3*	14.46	16.78	16.03	17.26		
R*	2.000	0.740	0.640	0.120		

Table 3. Orthogonal test results and range analysis.

Model item	Sum of squares	Df	F	F-value	Significance
Rotational speed	0.140	2	140.000	19.000	**
Temperature	0.120	2	120.000	19.000	**
pH	0.001	2	1.000	19.000	
Inoculation amount	0.021	2	21.000	19.000	*
Deviation	0.001	2			

Note: $F_{0.05}(2,2) = 19.0$; $F_{0.01}(2,2) = 99.0$; **: very significant; *: significant.

Table 4. Variance analysis of orthogonal.

Model item	Sum of squares	Df	F	F-value	Significance
Rotational speed	6.289	2	285.864	19.000	**
Temperature	0.866	2	39.364	19.000	*
pH	0.631	2	28.682	19.000	*
Inoculation amount	0.022	2	1.000	19.000	
Deviation	0.02	2			

$F_{0.05}(2,2) = 19.0$; $F_{0.01}(2,2) = 99.0$; **: very significant; *: significant.

4. Analysis and Discussion

The results show that temperature and rotational speed had a significant impact of extra cellular polysaccharides and *Agaricus blazei* Murill mycelium, it may be due to effects of bacteria in the fermentation process of intracellular enzyme activity and dissolved oxygen in fermentation of sake were temperature and speed, so these two factors were closely related to bacterial metabolism and growth. The other factors of affecting the fermentation have different effects on different fermentation process. Therefore we need to choose the optimal culture conditions in practical applications.

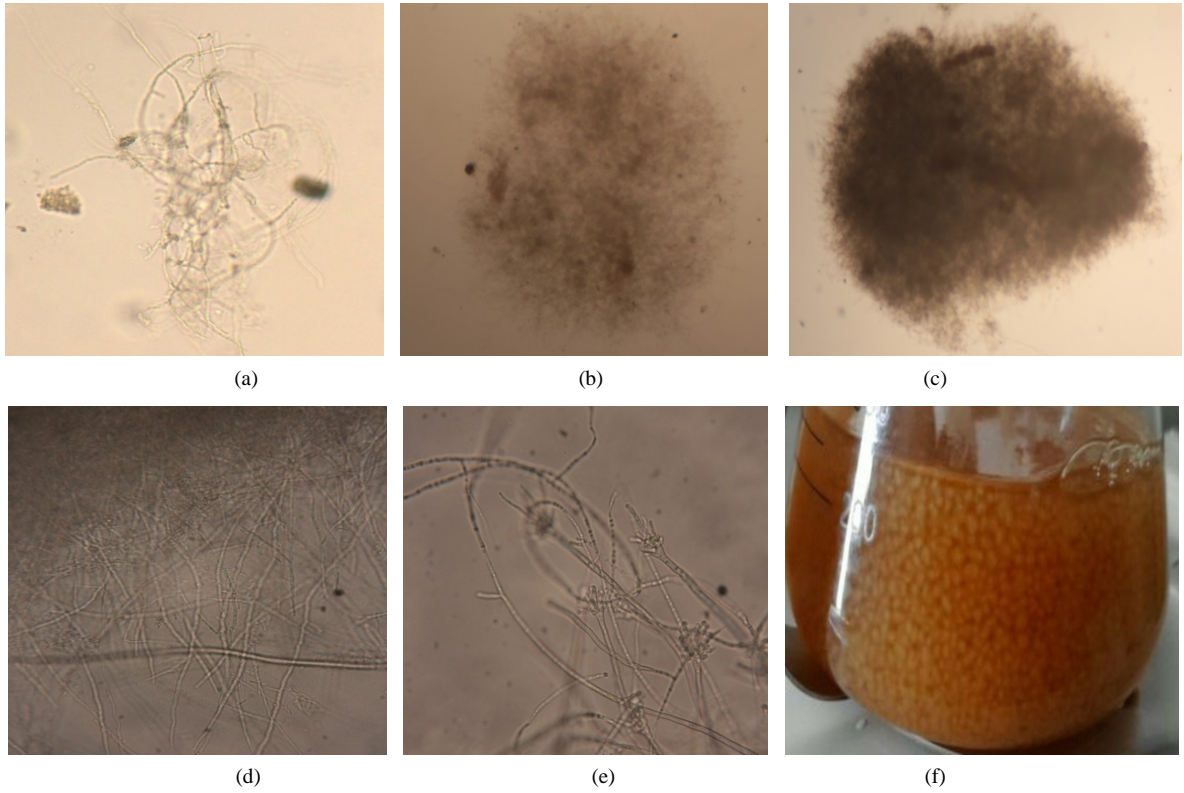
5. Conclusion

Through the research of bacterial liquid fermentation conditions of *Agaricus blazei* Murill, the results showed that different culture conditions on mycelium growth and extra cellular polysaccharide of *Agaricus blazei* Murill had a significant impact. When the culture conditions were at temperature (25°C), speed (150 r/min), pH (7) and vaccination (6%) maximum cell content, the mycelium dry weight was 1.32 g/100mL. When the training conditions were at temperature (25°C), rotational speed (150 r/min), pH (6.5) and vaccination (8%), the extra cellular polysaccharide content achieved maximum, and the extra cellular polysaccharide content reaches 6.95 mg/mL.

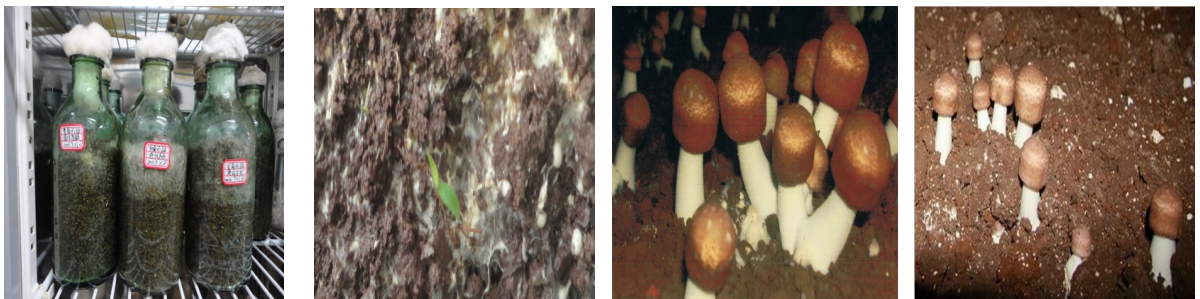
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Drawings



No 1. Morphology changes at different stage of *Agaricus blazei* Murill.



No 2. Sporocarp tests on liquid spawn of *Agaricus blazei* Murill.