

Full Length Research Paper

## The extraction on polysaccharide of sporocarp and static culture optimization conditions of *Morchella esculenta* from Qinghai-Tibetan Plateau

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In this paper, adopting orthogonal design method, the *Morchella esculenta* mycelia from Qinghai-Tibetan plateau was preliminary studied in different growing conditions such as pH, temperature, carbon sources, nitrogen sources and growth factors. The results showed that the *M. esculenta* mycelium liquid fermentation pH was between 6.0 to 7.0, cultivating optimum temperature 24°C, training required for 1.2% of potassium nitrate nitrogen (KNO<sub>3</sub>), carbon source was 4.5% soluble starch, growth factor biotin was 0.1%. At the same time, we also took the plateau *M. esculenta* polysaccharide fermentation liquid extraction, the extraction time was optimal combination 2 h, precipitated with ethanol concentration of 90% extraction ratio 1:20, the highest yield of polysaccharides.

**Key words:** Qinghai-Tibetan Plateau, *Morchella esculenta*, sporocarp, mycelium, static culture.

### INTRODUCTION

Polysaccharide is the material basis of life, it has a comparable information function of nucleic acid or protein, involved in cell-cell recognition, regulation of immune function and intercellular substance during transport, cell transformation, apoptosis, and most non-toxic. The research found that polysaccharides had anti-tumor and immunomodulatory (Zhang et al., 2007), anti-bacterial, anti-viral (Li et al., 2003), antioxidant and anti-aging, and many other pharmacological activities, it had become the ideal drug and natural sources of antioxidants. Polysaccharides exist in the mycelium, sporocarp and fermentation broth, which consists of more than 10 monosaccharide glycoside linkage to a polymer made of polymers

with complex biological activity and function. Among them, the most important one is immunomodulatory activity. Recently, fungal polysaccharides have been widely used into immunodeficiency diseases, autoimmune diseases, cancer and other diseases and clinical treatment and medical field for other purposes such as preparation of pharmaceutical materials, drug delivery agents, blood plasma substitutes. In recent years, the study of fungal polysaccharide and its compound has attracted more and more attention. So it has become one of the hot research areas such as molecular biology, medicine, food science, etc.

*Morchella esculenta* is famous for edible fungus with rich

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**Table 1.** The level table of  $L_9(3^4)$  on nutritional conditions

Level	Factor			
	A (%)	B (%)	C (%)	D (%)
1	0.3 KNO <sub>3</sub>	0.25 Biotin	10	2.5 C <sub>6</sub> H <sub>12</sub> O <sub>6</sub>
2	0.5 Peptone	0.5 VB <sub>2</sub>	20	3 Corn flour
3	0.3 Soy flour	0.75 IAA	30	3.5 Soluble starch

Note: The content of other components is respectively 0.5% KH<sub>2</sub>PO<sub>4</sub>, 0.1% MgSO<sub>4</sub> and 100mL H<sub>2</sub>O.

nutritious and delicious taste, which is welcomed by the international market. The content of amino acid lies on the first of all kinds of edible fungi. *M. esculenta* is internationally recognized as valuable medicinal fungus, its medicinal value of Chinese "broad spectrum of bacteria" and "compendium of materia medica" and other classics have been reported. Traditional medicine thought *M. esculenta* was natural, sweet and benefited the stomach and phlegm and lung airflow at ease. Modern medical research showed that *M. esculenta* polysaccharide is effective medicinal ingredients; it has enhanced immunity, anti-fatigue, anti-viral, tumor suppression and many other effects (Ren and Zhang, 1999).

In this article, on the basis of single factor experiment, static culture conditions were optimized and extraction technology of polysaccharide was selected adopting the orthogonal experiment. This research will provide the theoretical foundation for the antioxidant of natural polysaccharide development.

## MATERIALS AND METHODS

Static culture conditions optimized of *M. esculenta* mycelium.

### Test materials

**Tested strains:** the optimal use of screening strain Y2 (North mountain forestry farm, Huzhu, Qinghai, gathered on May 3, 2005, by isolating stalk of *Morchella vulgaris* to obtain the pure strain).

### Culture medium

Mother Culture Media: PDA integrated medium (potato 200 g, glucose 20 g, KH<sub>2</sub>PO<sub>4</sub> 1 g, MgSO<sub>4</sub>·7H<sub>2</sub>O 1 g, VB<sub>1</sub> 50 µg, agar 15 g, chloramphenicol 0.5 g), add distilled water to 1000 ml, pH 6.5, 121°C, sterilize 20 min.

**Liquid seed medium:** PDA medium without agar.

### Test method

Let the above medium become into plate medium, then take a different number of punch block *M. esculenta* mycelium, and take them to plant in the fresh medium (five repeats of each group). Finally place them in incubator.

### Determination on dry weight of colonies

Training 120 h, place the colonies and medium from the plates in a beaker with distilled water, each plate processing corresponds to a beaker, then the beaker is heated in the dry box. When the medium melted, mycelium will singled out with a sterile glass rod and the excess water was absorbed by the filter paper. Then place them in a weighing bottle and dry at 60°C until constant weight. At the end, they will be weighed (Wang et al., 2010). Based on the preliminary experiments of single factor, the orthogonal experiment was made by selecting the more important factor, including nitrogen (A), the type and concentration of growth factors (B), inoculum (C), carbon (D), pH (E), incubation time (F), incubation temperature (G), ventilation (H), impact on dry weight of *M. esculenta* mycelium. As there were more experimental factors and there are in order, the orthogonal experiment carried out twice (Wang, 2006). In the vicinity of the optimum value of each factor, three levels were taken and four factors and three levels orthogonal experiments were done with indicators for dry weight of mycelium. According to the  $L_9(3^4)$  cross-table, the technology of polysaccharide extraction was optimized (Tables 1 and 2).

### Polysaccharide extraction of *M. esculenta* fermentation liquor

#### Tested strains

The optimal screening strain Y2.

#### Media and culture conditions

Make media by applying the above, the best media and culture conditions obtained by the experiments.

#### Determination of polysaccharide content

*M. esculenta* fermentation broth was filtered after 21 days of culture. Then the filtrate was concentrated in a water bath at 90°C to the original volume of 1/3, slowly add 3 volumes of 80% ethanol solution, stand and overnight. The next day, the precipitation is extracted by centrifugation, dissolve in a small amount of hot water and wash three times with ethanol, centrifuge for 15 min with 3000 rpm. The precipitate was dried naturally in order to obtain polysaccharide fermentation broth of *M. esculenta*.

### Extraction technology of polysaccharide of *M. esculenta* sporocarp

#### Tested materials

*M. esculenta* sporocarp: collected from North mountain forestry farm of Huzhu in Qinghai Province.

**Table 2.** The level table of  $L_9(3^4)$  on culture conditions.

Level	Factor			
	E	F (h)	G (°C)	H
1	6.0	72	20	(1)
2	6.5	96	24	(2)
3	7.0	120	28	(3)

Note: (1) Three levels of ventilation volume were respectively 150, 250 and 350 ml can bottles with 100 ml culture medium. (2) The content of other components is respectively 0.5%  $KH_2PO_4$ , 0.1%  $MgSO_4$  and 100 ml  $H_2O$ .

**Table 3.** The orthogonal table of polysaccharide extraction of *Morchella esculenta* sporocarp.

Factor	Level		
	1	2	3
Extraction ratio (ml)	1:20	1:30	1:40
Extraction time (h)	2	4	6
Concentration of ethanol precipitation (%)	75	90	100

### Test Method

**Best extraction selection:** select three main factors impact on polysaccharide extraction of *M. esculenta* sporocarp including extraction ratio, extraction time and ethanol concentration. The orthogonal experiment was made by using  $L_9(3^4)$  and regarding the ratio of *M. esculenta* polysaccharide as investigated indicators (Table 3).

**Extraction method:** dry *M. esculenta* sporocarp in 60°C and grind them for used. Take 9 parts of *M. esculenta* sporocarp with the weight of 10 g, then place them into 1000 ml beaker, which was added 20 times with distilled water (200 ml), 30 times (300 ml), 40 times (400 ml), and finally take them into thermostatic water bath with the water temperature controlled at about 95°C. They were extracted with water volume and extraction time under orthogonal test. Centrifuge for 10 min with 4000 r/min speed, take the supernatant, extract precipitate again by the above method, concentrate them, the above extract was concentrated according to orthogonal 3-fold volume, added various concentrations of ethanol precipitation, overnight, discarded the supernatant liquid centrifugal, derived from sediments into the Petri dish, dried at 60°C in the roasting oven, and get morel fruiting polysaccharides (Meng et al., 2013).

## RESULTS AND ANALYSIS

### Static culture conditions optimized of *M. esculenta* mycelium

Through the intuitive analysis of data (Tables 4 and 6), the static culture conditions of *M. esculenta* mycelium was selected out by orthogonal test. Namely, when use water as solvent and take dry weight as evaluation indicators, the best mix programme was  $A_1B_2C_2D_3$  and  $E_2F_3G_2H_2$ . What is more, 0.3%  $KNO_3$ , 0.5%  $VB_2$ , 10% vaccination volume, 3.5% soluble starch as carbon source, pH 6.5, 120min training time, the most suitable temperature for 24°C and ventilation volume for 250 ml. Analysis of variance

table (Tables 5 and 7) showed that the impact extent order of various factors on dry weight of mycelium is: carbon source > growth factor > nitrogen > inoculation, incubation temperature > pH > incubation time > ventilation. The carbon source, growth factors, nitrogen, temperature, pH and incubation time have significantly effect on the dry weight of *M. esculenta*.

### Study on extraction technology of polysaccharide from *M. esculenta* sporocarp

The results were shown in Table 8. When the ratio of extraction was 1:30, extraction time was 2 h and concentration of ethanol was 90%, the production of polysaccharides yield was the highest and reached for 13.1%, which was basically consistent with the result reported by Wu and An (Wu and An, 2005), but it was lower than Li and Qiu (Li and Qiu, 2005) who reported. Thus, we thought that it was a certain relationship with the extraction method of polysaccharide fermentation liquid. Li and Qiu (Li and Qiu, 2005) used a method of enzyme extraction, so extraction yield of polysaccharide could be increased significantly through this method. They determined the optimum conditions of pectinase action were 15% enzyme dosage, 50°C and 3 h and the optimal conditions of cellulase enzyme were 15% enzyme dosage, 45°C and 3 h. But it could be seen from the tables, the polysaccharides yield is little difference when extraction ratio were 1:20, 1:30 and 1:40. If considering the practicality of technology, the choice of 1:20 was the best extraction ratio. Extraction time had the greater impact on the extraction of polysaccharide. When extraction time was 2 h, the average yield of polysaccharide was the highest, so 2 h should be used for the best extraction time. At the same

**Table 4.** The design scheme and results of orthogonal test  $L_9 (3^4)$  about medium formula of *Morchella esculenta*.

Number	Factor		Dry weight of mycelium (mg/100ml)					Sum	Average value (mg/100ml)
	A	B	C (%)	D (%)	I	II	III		
1	1 (0.3% KNO <sub>3</sub> )	1(0.25% Biotin)	1(10)	1(2.5% C <sub>6</sub> H <sub>12</sub> O <sub>6</sub> )	217.3	210.4	213.2	640.9	213.6
2	1	2 (0.5%VB <sub>2</sub> )	2(20)	2(3% Corn flour)	205.6	214.3	203.2	623.1	207.7
3	1	3(0.75% IAA)	3(30)	3(3.5% Soluble starch)	215.4	207.3	209.6	632.3	210.8
4	2 (0.5% Peptone)	1	2	3	220.0	218.4	210.6	649	216.3
5	2	2	3	1	204.6	212.3	217.5	634.4	211.5
6	2	3	1	2	178.3	186.2	179.4	543.9	181.3
7	3 (0.3% Soy flour)	1	3	2	185.4	190.2	196.3	571.9	190.6
8	3	2	1	3	207.1	203.1	210.8	621	207.0
9	3	3	2	1	196.3	189.7	192.4	578.4	192.8
T <sub>1</sub>	1896.3	1861.8	1805.8	1853.7					
T <sub>2</sub>	1827.3	1878.5	1850.5	1738.9					
T <sub>3</sub>	1771.3	1754.6	1838.6	1902.3					
X <sub>1</sub>	210.7	206.8	200.6	206.0					
X <sub>2</sub>	203.0	208.7	205.6	193.2					
X <sub>3</sub>	196.8	195.0	204.3	211.4					
R	13.9	13.8	5.0	18.2					
Optimization parameters	A <sub>1</sub>	B <sub>2</sub>	C <sub>2</sub>	D <sub>3</sub>					

**Table 5.** The variance table of orthogonal experiment on *Morchella esculenta* medium formulation.

Source of variation	SS	df	MS	F	P
Block	3559.26	8	444.91	19.53	<0.001
A	871.19	2	435.59	19.12	<0.001
B	1004.52	2	502.26	22.05	<0.001
C	119.09	2	59.55	2.61	0.1
D	1564.47	2	782.23	34.34	<0.001
The experimental error	409.97	18	22.78		
The total variation	3969.23	26	152.66		

time, ethanol concentration was 90%, the average yield of polysaccharide was the highest, so it was the best to select 90% ethanol precipitation. As could be seen from the above results, the optimum combination of extraction of *M. esculenta* polysaccharide was that, extraction time was 2 h, 90% ethanol precipitated and extraction ratio was 1:20. Meanwhile, by orthogonal analysis of variance table (Table 9) showed that it had reached significantly that ethanol concentration had effected on the yield of polysaccharide.

## Conclusion

*M. esculenta* sporocarp growing on the ecological environment requirements was extremely strict and has obvious season. Because mycelium and sporocarp of *M. esculenta* were different, they were not the same with demand for nutrients, temperature, pH, moisture, light, oxygen and so on. In different nutritional conditions, the shape, color and other cultural characteristics of myce-

lium, which were growing in the same strain of *M. esculenta*, are not identical. These differences reflected the diversity of physiological characteristics about *M. esculenta*. This diversity caused the volatility of domesticated conditions and increased the difficulty of domestication. At present, the artificial cultivation could still not achieve commercialized cultivation and wild resources of *M. esculenta* was very limited, but the demand for *M. esculenta* became more and more, so we must find another way in order to do the sustainable development and utilization of *M. esculenta* resources. Through the experiment, we found that the nutrients contained in mycelium produced by liquid fermentation were basically consistent with those from sporocarp, so mycelium and mycelium polysaccharide should be obtained by the massively artificial liquid fermentation and those products were developed and utilized. Because Qinghai province had the unique geographical environment and location, and *M. esculenta* resources were rich and various, so the wild resources of *M. esculenta* were realized sustainable development. On

**Table 6.** The orthogonal test ( $L_9(3^4)$ ) design scheme and results of *Morchella esculenta* culture conditions.

Number	Factor			Dry weight of mycelium (mg/100ml)			Sum	Average value mg/100ml	
	E	F (h)	G (°C)	H(ml)	I	II			III
1	1(6.0)	1(72)	1 (20)	1(150)	164.3	172.1	168.4	504.8	168.3
2	1	2(96)	2 (24)	2(250)	177.2	179.5	182.3	539	179.7
3	1	3(120)	3 (28)	3(350)	160.4	173.6	168.2	502.2	167.4
4	2(6.5)	1	2	3	185.4	190.7	179.6	555.7	185.2
5	2	2	3	1	167.2	173.3	178.1	518.6	172.9
6	2	3	1	2	193.5	187.2	191.6	572.3	190.8
7	3 (7.0)	1	3	2	158.4	167.2	170.2	495.8	165.3
8	3	2	1	3	180.1	186.2	178.3	544.6	181.5
9	3	3	2	1	190.7	186.4	193.2	570.3	190.1
T <sub>1</sub>	1546	1556.3	1621.7	1593.7					
T <sub>2</sub>	1646.6	1602.2	1725	1607.1					
T <sub>3</sub>	1610.7	1644.8	1516.6	1602.5					
X <sub>3</sub>	179.0	182.8	168.5	178.0					
R	11.2	9.8	16.5	1.5					
Optimization parameters	E <sub>2</sub>	F <sub>3</sub>	G <sub>2</sub>	H <sub>2</sub>					

**Table 7.** Analysis of variance table of orthogonal experiment on *Morchella esculenta* culture conditions.

Sources of variation	SS	df	MS	F	P
Block	2317.43	8	289.68	12.81	<0.001
E	577.60	2	288.80	12.77	<0.0001
F	435.33	2	217.66	9.63	<0.0001
G	1294.20	2	647.10	28.62	<0.001
H	10.30	2	5.15	0.23	0.7985
Experimental error	406.97	18	22.61		
Total variation	2724.40	26	104.78		

**Table 8.** The experimental results and intuitive analysis on polysaccharide extraction of *Morchella esculenta* sporocarp.

Number	Extraction ratio	Extraction time (h)	Concentration of ethanol precipitation (%)	Polysaccharide production (g)	Polysaccharide yield (%)
	A	B	C		
1	1	1	1	1.21	12.1
2	1	2	2	1.20	12.0
3	1	3	3	1.24	12.5
4	2	1	2	1.32	13.1
5	2	2	3	0.70	7.0
6	2	3	1	1.10	11.1
7	3	1	3	1.20	12.0
8	3	2	1	1.05	10.5
9	3	3	2	1.09	12.9
K <sub>1</sub>	1.217	1.243	1.120		
K <sub>2</sub>	1.040	0.983	1.203		
K <sub>3</sub>	1.113	1.143	1.047		
R	0.177	0.260	0.156		
Optimization parameters	A <sub>1</sub>	B <sub>1</sub>	C <sub>2</sub>		

**Table 9.** Analysis of variance table of orthogonal experiment on polysaccharide extraction.

Source of variation	SS	df	MS	F	P
Block	0.19	6	0.03	0.89	0.62
A	0.05	2	0.02	0.67	0.60
B	0.10	2	0.05	1.46	0.41
C	0.04	2	0.02	0.52	0.66
The experimental error	0.07	2	0.04		
Total variance	0.26	8	0.03		

On the one hand, liquid fermentation of *M. esculenta* were studied and related products were developed in order to research and develop mycelium polysaccharides and related products; On the other hand, the relevant departments should formulate corresponding policies to manage and control the collection, purchase, sales, and actively establish production and experimental base for the implementation of artificial and artificial cultivation so as to achieve the purpose of effective protection and rational utilization of *M. esculenta* resources and realize sustainable development.

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