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Effect of nitrogen concentration in culture mediums on growth and enzyme production of *Phanerochaete chrysosporium*

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Abstract: Effect of different nitrogen concentration in the mediums on growth and enzyme production of *Phanerochaete chrysosporium* was studied when glucose concentration was 10 g/L. The results showed that the medium contained 0.8 g/L ammonium tartrate is the best. It not only supply abundant nutrients for the growth of *Phanerochaete chrysosporium*, which make mycelia the best grow compared with the other medium, but also produce higher manganese-dependent peroxidase(Mnp) and laccase(Lac) activity. In addition, it is observed that the variation of mycelia surface is related to ligninolytic enzyme secreted by *Phanerochaete chrysosporium*. When the surface of mycelium pellets appeared burs, it predicts secondary metabolism begin. This experimentation demonstrated that when the ratio of carbon and nitrogen in nitrogen limited medium is equal to 100:8, growth and enzyme production of *Phanerochaete chrysosporium* is the best, it could achieve the maximum Mnp and Lac activity.

Keywords: white rot fungus; *Phanerochaete chrysosporium*; nitrogen concentration; manganese-dependent peroxidase(Mnp); laccase (Lac)

Introduction

With the development of society, the amount and sort of synthetic chemicals sharply increase. Some of these synthetic chemicals are hardly removed with traditional physical, chemical and biological method. In order to solve increasingly outstanding environmental problems, some new technologies and methods were studied. In 1987, Kirk *et al*. observed that lignin-degrading enzymes secreted by white rot fungi during secondary metabolism have nonspecific characteristics, i.e. they not only can degrade lignin but also can degrade many xenobiotic matter and toxic organo-pollutants (Kirk, 1987). White rot fungi can secrete peroxidase to degrade xenobiotic compounds under carbon and/or nitrogen limitation(Keyser, 1978; Jeffries, 1981; Thurston, 1994), so it will more and more be used in water pollution control and soil bioremediation.

The white rot fungi *Phanerochaete chrysosporium* had the ability to degrade, besides lignin, a wide spectrum of recalcitrant organo-pollutants, so it has now become a model for many studies on biodepollutions (Zouari, 2002; Shim, 2002; Kapich, 2004). *Phanerochaete chrysosporium* produces several extracellular peroxidases, including lignin peroxidases(Lip; Tien, 1983), manganese-dependent peroxidase(Mnp; Renganathan, 1985), and laccase(Lac; Srinivasan, 1995) during secondary metabolism in response to carbon and/or nitrogen starvation. So the amount of carbon and nitrogen in the medium play an important role in growth and enzyme production of *Phanerochaete chrysosporium*. However, at present, many studies on *Phanerochaete chrysosporium* only limited to two kinds of culture mediums given by Tien and Kirk, i.e. N-limited medium (C/N = 56/2.2 mmol/L) and C-limited medium(C/N = 28/44 mmol/L). Then what is the ratio of carbon and nitrogen in the medium, the growth and enzyme production of *Phanerochaete chrysosporium* is optimal? Up to now, no literature is reported.

The purpose of this work was to study growth and enzyme production of *Phanerochaete chrysosporium* under different nitrogen concentration in the medium and determine the optimal ratio of carbon and nitrogen in the medium during incubation.

1 Materials and method

1.1 Fungal strains

Phanerochaete chrysosporium BKM-F-1767 was obtained from Guangzhou Institute of Chemistry, Chinese Academy of Sciences. The fungus was maintained on plates of potato dextrose agar at 4℃.

1.2 Medium

1.2.1 Solid medium

Solid medium used is media containing potato lixiviums 200 g/L, glucose 20 g/L and agar 20 g/L.

1.2.2 Liquor medium

The growth medium was prepared according to Tien and Kirk (Tien, 1988), and medium compositions are as follows: glucose 10 g/L; KH_2PO_4 2.0 g/L; $MgSO_4$ 0.5 g/L; $CaCl_2$ 0.1 g/L; 20 mmol/L acetate buffer(pH 4.4); 1.5 mmol/L veratryl alcohol; trace elements 70.0 ml/L. This trace elements solution contained: $MgSO_4$ 0.21 g/L; $MnSO_4$ 35 mg/L; NaCl 70 mg/L; $FeSO_4 \cdot 7H_2O$ 7 mg/L; $CoCl_2$ 7 mg/L; $ZnSO_4 \cdot 7H_2O$ 7 mg/L; $CuSO_4$ 7 mg/L; $AlK(SO_4)_2 \cdot 12H_2O$ 0.7 mg/L; H_3BO_3 0.7 mg/L; $Na_2MoO_4 \cdot 2H_2O$ 0.7 mg/L; nitrilotriacetate 0.105 g/L.

In order to obtain the liquor medium containing different nitrogen concentration, the concentration of ammonium tartrate in the medium was 0.0 g/L; 0.2 g/L; 0.4 g/L; 0.8 g/L; 2.0 g/L; 4.0 g/L; 10.0 g/L, respectively. Before inoculation vitamin B1 was injected under sterile condition, and its concentration was 1 mg/L.

1.3 Cultivation condition

The white rot fungus was inoculated on PDA medium for 5—7 d at 32℃. Then, spore was harvested in sterile deionized water by gently scraping the surface of the culture with a sterile inoculating loop and filter-sterilized. The spore suspension was inoculated in 250 ml Erlenmeyer flasks with 100 ml of liquor medium. The inoculated spores concentration was adjusted to 10^5 spores/ml and used as an inoculum for further studies. The Erlenmeyer flasks were incubated under agitation (160 r/min) at 37℃. Experiments were done in duplicate and samples were analysed in triplicate, and the results were expressed as the mean values.

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1.4 Analytical methods

1.4.1 Chemicals

Veratryl alcohol, 2,2'-azinobis-ethylbenzthiazoline (ABTS) and nitrilotriacetate were from Fluka Company (Buchs, Switzerland). The other chemicals used were of analytical grade.

1.4.2 Preparation of crude ligninolytic enzymes

The crude ligninolytic enzymes were harvested by centrifuging at 9000 r/min for 10 min.

1.4.3 Enzyme assays

Lignin peroxidase (Lip) activity was determined spectrophotometrically at 310 nm according to Tien and Kirk (Tien, 1988). One unit(U) was defined as the amount of enzyme that oxidized 1 μmol of veratryl alcohol to veratraldehyde in 1 min, and the activities were reported as U/L.

Manganese-dependent peroxidase (Mnp) activity was estimated according to Paszczynski *et al*. (Paszczynski, 1988). One unit(U) was defined as the amount of enzyme that oxidized 1 μmol of Mn^{2+} to Mn^{3+} in 1 min, and the activities were reported as U/L.

Laccase (Lac) activity was determined spectrophotometrically as described by Bourbonnais and Paice (Bourbonnais, 1990). One unit(U) was defined as the amount of enzyme that oxidized 1 μmol of ABTS per minute, and the activities were expressed in U/L.

1.4.4 Mycelia dry weight determination

The dry weight of the fungal mass, expressed as milligram of biomass per 100 milliliter of culture, was determined by filtering the content of each flask when the mycelium pellets were grown for 6 d and drying to a constant weight at 105℃.

2 Results and discussion

2.1 Effect of different nitrogen concentration on growth of *Phanerochaete chrysosporium*

Any microorganism needs to obtain nutrients from environment for its reproduction and growth. White rot fungi *Phanerochaete chrysosporium* is the similar too. Its growth must also depend on nutrients. Carbon and nitrogen are the main elements in cell of white rot fungi *Phanerochaete chrysosporium*. So carbon source and nitrogen source play an important role in the whole nutrients. Based on this, the effect of different nitrogen concentration on the growth of *Phanerochaete chrysosporium* was studied when fixing carbon concentration in medium. The purpose of this study was to investigate the amount of nitrogen source that *Phanerochaete chrysosporium* requires during growth when the concentration of carbon source is not changeable. It will build theoretical foundation for optimizing the compositions of culture medium.

2.1.1 Size of mycelium pellets

Except for the liquor medium without adding ammonium tartrate, the mycelium pellets were produced in the other liquor mediums contained *Phanerochaete chrysosporium*, which were incubated under agitation(160 r/min) at 37℃ after 1 d, and the size and amount of mycelium pellets were equivalent. However, with continuing incubation, the size and amount of mycelium pellets in different nitrogen concentration liquor medium were different. The amount of mycelium pellets greatly increased with the rise of ammonium tartrate concentration in the medium, but the diameter of mycelium pellets did not increase. Fig.1 shows the variation of diameter of mycelium pellets incubated after 5 d. It can be shown that there is a extremum point at the curve, namely, the diameter of mycelium pellets are the biggest when the concentration of ammonium tartrate in the medium is 0.8 g/L, and they are about 5 mm. On the contrary, if the concentration of ammonium tartrate in the medium is over 0.8 g/L, the diameter of mycelium pellets gradually decreases with the increase of concentration of ammonium tartrate.

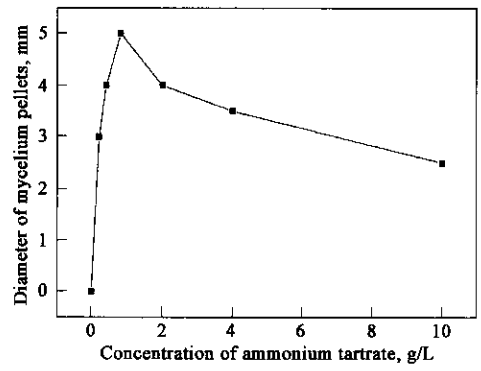

Fig.1 Variation of diameter of mycelium pellets with the concentration of ammonium tartrate

The results showed that *Phanerochaete chrysosporium* cannot grow without nitrogen in the medium, at the same time, the excessive nitrogen is also able to restrict the growth of *Phanerochaete chrysosporium*. Thus, it is very important to select appropriate ratio of carbon and nitrogen when white rot fungus *Phanerochaete chrysosporium* is incubated.

2.1.2 Surface of mycelium pellets

The surface of mycelia appeared a series of variation during incubation of *Phanerochaete chrysosporium*. At initial stages of incubation, the surface of mycelium pellets is smooth. With continuing incubation, the surface of mycelium pellets begins to produce many burs. Again, continuing incubation, some burs in the surface of mycelium pellets begin to fall off, so it makes liquor medium turbid. Time of appearing or falling off the burs is various with the difference of nitrogen concentration in the medium. Results are shown in Table 1. There is not mycelium pellets in liquor medium with 0 g/L ammonium tartrate, so its data are not listed in Table 1. The results also testify nitrogen source is absolutely necessarily for the growth of *Phanerochaete chrysosporium*.

Table 1 Variation of the surface of mycelium pellets in different nitrogen concentration medium during incubation

Nitrogen in the medium	Incubation time, d								
	1	2	3	4	5	6	7	8	9
0.2 g/L	-	-	±	+	+	+	O	O	O
0.4 g/L	-	-	±	+	+	+	O	O	O
0.8 g/L	-	-	±	+	+	+	O	O	O
2.0 g/L	-	-	-	+	O	O	O	O	O
4.0 g/L	-	-	-	±	O	O	O	O	O
10.0 g/L	-	-	-	-	±	O	O	O	O

Notes: the surface of mycelium pellets is smooth(-); the surface of mycelium pellets is between smooth and appearing burs(±); burs in the surface of mycelium pellets appear(+); burs in the surface of mycelium pellets fall off(O)

Table 1 shows that the concentration of ammonium tartrate is the less, time of appearing burs in the surface of mycelium pellets is the less, and the time of shelling burs will lag. When the concentration of ammonium tartrate in the medium is over 2.0 g/L, the time of appearing burs not only greatly lag, but also some burs appeared just now already begin to fall off. The reason caused the phenomenon is related to the secondary metabolism.

In fact, burs that the surface of mycelium pellets grows are filamentous mycelia. The surface of mycelium pellets appeared a great deal filamentous mycelia is related to the exhaustion of nutrients (including nitrogen or carbon). Because filamentous mycelia have huge specific surface area, compared with mycelium pellets. It is propitious to

compete nitrogen source. In addition, *Phanerochaete chrysosporium* began to produce enzyme when the nutrients in reaction system become limitation(Keyser, 1978). So the burs in surface of mycelium pellets also indicated the appearance of secondary metabolism.

2.1.3 Weight of mycelium pellets

In order to further appraise the effect of nitrogen concentration in the medium on growth of *Phanerochaete chrysosporium*, and find out the optimal C/N ratio in the medium for growth of *Phanerochaete chrysosporium*. On the basis of the above experiment, the mycelium pellets incubated for 6 d were dried and weighed. Results are shown in Fig.2.

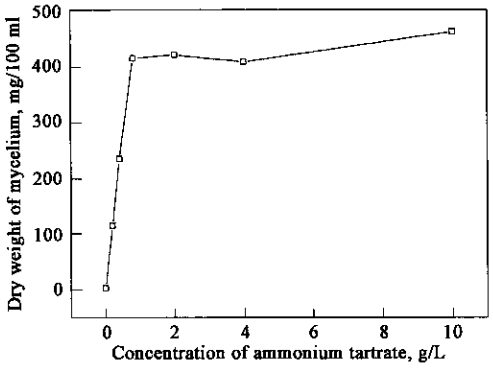

Fig.2 Growth of *Phanerochaete chrysosporium* in different nitrogen concentration

Fig.2 shows that the variation of dry weight of mycelia with the rise of concentration of ammonium tartrate in the medium. When the concentration of ammonium tartrate in the medium was 0.8 g/L, the dry weight of mycelia incubated after 6 days reached to 415 mg/100 ml. However, when continuing to raise the concentration of ammonium tartrate, the dry weight of mycelia increased slowly. As a result, when the concentration of glucose in the medium is 10 g/L, the concentration of ammonium tartrate should be selected 0.8 g/L, in other words, the ratio of glucose and ammonium tartrate should be 100:8.

Thus, nitrogen source is very important for the growth of *Phanerochaete chrysosporium*. And there is an optimal value in the concentration of nitrogen source. According to the theory of microbiology, the optimal value is related to the concentration of carbon source in the medium, namely, the amount of carbon and nitrogen in the medium should be proportionate for the growth of *Phanerochaete chrysosporium*. If the ratio of carbon and nitrogen is appropriate, it is prone to the growth of *Phanerochaete chrysosporium*, or else, it may be inhibit the growth of *Phanerochaete chrysosporium*.

2.2 Effect of different nitrogen concentration on enzyme production of *Phanerochaete chrysosporium*

The ligninolytic enzymes of *Phanerochaete chrysosporium* are secreted when carbon and/or nitrogen becomes limiting. Based on the above experiments, the effect of different nitrogen concentration on enzyme production of *Phanerochaete chrysosporium* was studied.

Manganese peroxidase(Mnp) and the laccases were only measured throughout the incubation of *Phanerochaete chrysosporium* for 9 d. However, no lignin peroxidase(Lip) was observed. The reason is that the whole experiment was performed under nitrogen limitation. This result accords with the opinion of Rothschild *et al*., namely, lignin peroxidase was easily produced under carbon limitation (Rothschild, 1995). But it is opposite with that of Faison's(Faison, 1985). So the production mechanism of lignin peroxidase should be studied further.

2.2.1 Manganese peroxidase(Mnp)

Fig.3 shows the effect of different nitrogen concentration on the production of manganese peroxidase. Manganese peroxidase can obviously be measured under lower the concentration of ammonium tartrate such as 0.2 g/L and 0.8 g/L. However, the time appeared the highest enzyme activity was different at the two conditions. The fewer concentration of ammonium tartrate, the shorter time appeared the highest enzyme activity. The concentration of ammonium tartrate is 0.2 g/L, the highest value of Mnp activity was achieved on the day 6(481.6 U/L). And that the concentration of ammonium tartrate is 0.8 g/L, the highest value of Mnp activity was achieved on the day 8(535.5 U/L). This rule is related to the mechanism of enzyme production of *Phanerochaete chrysosporium*. Because the manganese peroxidase was not secreted by *Phanerochaete chrysosporium* until carbon and/or nitrogen source were exhausted or were close to be exhaust(Yu, 2003). When the amount of fungal inoculum is equivalent, the lower nitrogen concentration in the medium, the shorter time being exhausted is. But, if no ammonium tartrate is added in the medium, not only manganese peroxidase was not measured, but also mycelium pellets were not produced. In addition, manganese peroxidase production is very low under high concentration of ammonium tartrate(2 g/L—10 g/L). The reason is that there is residual nitrogen in reaction system, and they inhibited the production of peroxidase.

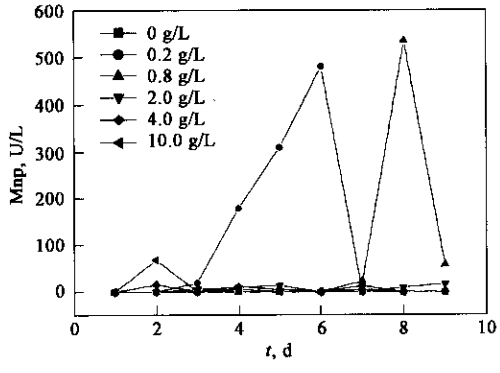

Fig.3 Effect of different nitrogen concentration on production of Mnp

2.2.2 Laccase(Lac)

Fig.4 shows the effect of different nitrogen concentration on the production of laccase. Similar to production of manganese peroxidase, laccase was also produced under lower nitrogen concentration, and that no laccase was observed under higher nitrogen concentration(2 g/L—10 g/L). At the same time, with the rise of nitrogen concentration in the medium, the time that appeared the highest value of Lac activity will prolong. When the concentration of ammonium tartrate was 0.2 g/L, 0.4 g/L and 0.8 g/L, the maximum enzyme activity was achieved on the day 5, 6 and 8, respectively, and its value was 4.07 U/L, 8.93 U/L and 20.76 U/L. The reason is similar to that of manganese peroxidase.

2.2.3 Peak value of Mnp and Lac activity under different nitrogen concentration

Fig.5 shows peak value of Mnp and Lac activity under different nitrogen concentration. It has been shown that the effect of the nitrogen concentration on production of Mnp was similar to that of Lac. Low nitrogen concentration is propitious to produce Mnp and Lac, whereas high nitrogen concentration inhibits their production. It is very interesting that peak value of Mnp and Lac activity were reached simultaneously when the concentration of ammonium tartrate was 0.8 g/L. So, if the concentration of glucose in the medium was 10 g/L, selecting 0.8 g/L ammonium tartrate should be optimal. Moreover, Mnp and Lac activity could all reach to the maximum.

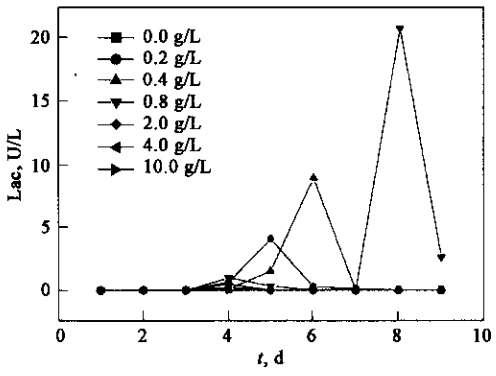

Fig.4 Effect of different nitrogen concentration on production of Lac

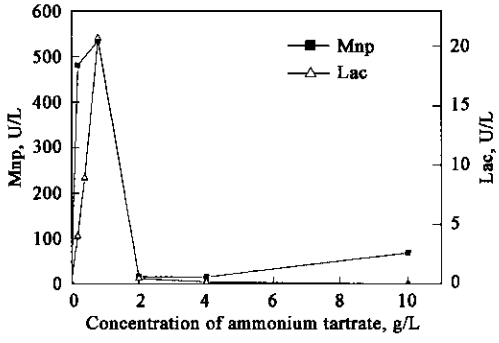

Fig.5 Variation of peak value of Mnp and Lac activity under different nitrogen concentration

In sum, the nitrogen concentration in the medium directly influences enzyme production of *Phanerochaete chrysosporium*. In general, the low nitrogen concentration in the medium is propitious to produce Mnp and Lac. But, if nitrogen concentration is very low, it easily makes the enzyme activity decline. So, the ratio of carbon and nitrogen in the medium should be appropriate for obtaining optimal culture medium. According to the results of this experiment, the ratio of carbon and nitrogen should be 100:8 for producing the maximum Mnp and Lac activity.

3 Conclusions

When the concentration of glucose in the medium is invariable, nitrogen concentration plays an important role in the growth and enzyme production of *Phanerochaete chrysosporium*. Not only high nitrogen concentration does not increase the mycelia, but also inhibits the production of manganese peroxidase and laccase. Although the low nitrogen concentration is propitious to the production of Mnp and Lac, low nutrient makes the amount of mycelia decrease, thereafter it makes the enzyme activity fall off.

When the concentration of glucose in the medium is invariable, both the nitrogen concentration in the medium and the time that the maximum enzyme activity appeared is positive correlation. Only nitrogen in the medium was exhausted by *Phanerochaete chrysosporium*, enzyme activity appeared. So the amount of fungal incubation is fixed, the time that the maximum enzyme activity appeared will prolong with the addition of nitrogen concentration.

In order to obtain the maximum amount of manganese peroxidase and laccase under nitrogen limitation, the ratio of carbon and nitrogen in the medium should be 100:8.

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