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Effects of culture conditions on ligninolytic enzymes and protease production by *Phanerochaete chrysosporium* in air

XIONG Xiaoping, WEN Xianghua*, BAI Yanan, QIAN Yi

State Key Joint Laboratory of Environmental Simulation and Pollution Control, Department of Environmental Science and Engineering, Tsinghua University, Beijing 100084, China. E-mail: xxp02@mails.tsinghua.edu.cn

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Abstract

The production of ligninolytic enzymes and protease by *Phanerochaete chrysosporium* was investigated under different culture conditions. Different amounts of medium were employed in free and immobilized culture, together with two kinds of medium with different C/N ratios. Little lignin peroxidase (LiP) (< 2 U/L) was detected in free culture with nitrogen-limited medium (C/N ratio: 56/2.2, in mmol/L), while manganese peroxidase (MnP) maximum activity was 231 and 240 U/L in 50 and 100 ml medium culture, respectively. Immobilized culture with 50 ml nitrogen-limited medium gave the highest MnP and LiP production with the maximum values of 410 and 721 U/L separately on the day 5; however, flasks containing 100 ml nitrogen-limited medium only produced less MnP with a peak value of 290 U/L. Comparatively, carbon-limited medium (C/N ratio: 28/44, in mmol/L) was adopted in culture but produced little MnP and LiP. Medium type had the greatest impact on protease production. Large amount of protease was produced due to glucose limitation. Culture type and medium volume influence protease activity corporately by affecting oxygen supply. The results implied shallow immobilized culture was a possible way to gain high production of ligninolytic enzymes.

Key words: protease; culture conditions; ligninolytic enzymes; *Phanerochaete chrysosporium*

Introduction

The white rot fungus *Phanerochaete chrysosporium* has been extensively studied because of its powerful ligninolytic enzymes. These enzymes, mainly including lignin peroxidase (LiP) and manganese peroxidase (MnP), are secreted during the secondary metabolism triggered by carbon, nitrogen or sulfur limitation (Jeffries *et al*., 1981; Tien and Kirk, 1983, 1988). They have been demonstrated to play a crucial role in lignin degradation and showed great potential in paper industry. At the same time, more and more researches have revealed that the ligninolytic enzymes are nonspecific enzymes and can assist in the degradation of a wide variety of recalcitrant organic pollutants, such as polycyclic aromatic hydrocarbons (PAHs), pesticide and dyes, as has been reviewed by Cameron *et al*. (2000). This raised the interest of study in ligninolytic enzymes production.

To realize application of ligninolytic enzymes, a large production of the biocatalysts at low cost is needed (Cabaleiro *et al*., 2002). However, most laboratory studies have been conducted in pure oxygen or in an oxygen-enriched environment (Dosoretz *et al*., 1990a; Zhen and Yu, 1998), which increased the production cost. An effective synthesis of ligninolytic enzymes in air would imply lower cost and greater feasibility essential for their large scale production (Yu *et al*., 2006). So far, successful culture of *P. chrysosporium* with high ligninolytic enzymes production in air has rarely been reported. On the other hand, significant losses of enzyme activity occurred during all cultivations, which prevent enzymes accumulation in crude fermentation product. The simultaneous secretion of proteolytic enzymes (protease) could have caused the low stability of produced peroxidases although different viewpoints still exist (Cabaleiro *et al*., 2001, 2002; Chung *et al*., 2005; Dass *et al*., 1995; Dosoretz *et al*., 1990b, c; Jimenez *et al*., 2003; Pascal *et al*., 1993). Further study about protease production and its relationship with ligninolytic enzymes production in different culture conditions is needed to assist in directing fermentation process design.

In the present report, the fermentation was carried out in batches under air atmosphere. Different volumes of both carbon-limited and nitrogen-limited medium were tested in both free and immobilized cultures. Protease and ligninolytic enzymes were measured during the whole fermentation process. Later, the effects of culture conditions, including culture type (free or immobilized), medium species (carbon-limited or nitrogen-limited) and volumes, on protease production, as well as the relationship between protease and ligninolytic enzymes, were discussed based on the results.

*Corresponding author. E-mail: xhwen@tsinghua.edu.cn.

1 Materials and methods

1.1 Strain

Phanerochaete chrysosporium strain BKM-F-1767 (ATCC 24725) was maintained at 37°C on PDA plates.

1.2 Carriers

Polyurethane foam cubes of 5-mm per side (Dongfang Polyurethane Foam Co., Beijing, China) were employed as the support in immobilized cultures. Prior to use, they were treated by boiling for 10 min and washing thoroughly three times with distilled water. After that, the carriers were dried at room temperature overnight and autoclaved at 121°C for 20 min (Couto *et al*., 2002a).

1.3 Culture conditions

The nitrogen-limited medium was prepared based on that described by Tien and Kirk (1988) with 10 g/L glucose as carbon source, except that the dimethylsuccinate was replaced by 20 mmol/L acetate buffer (pH 4.5). Veratryl alcohol 1.5 mmol/L was introduced at the beginning of cultures and no surfactant was added (Couto and Ratto, 1998). Seven-day-old spores were harvested in sterilized water, filtered through glass-wool and adjusted to absorbance of 0.5 at 650 nm. This spore suspension (about 2.5×10^6 spores/ml) was used for inoculation (Urek and Pazarlioglu, 2004).

In carbon-limited medium, C/N ratio was altered from 56/2.2 (in mmol/L nitrogen-limited medium) to 28/44 (in mmol/L) (Yu *et al*., 2005) and the other components were the same.

1.3.1 Free cultures

Two series of medium volume (100 ml, 50 ml) were adopted in 250-ml Erlenmeyer flasks, 100-ml and 50-ml. When 100 ml medium was added, 4 ml spore suspension (maintained before) was used for inoculum and half of that was added in the flasks with 50 ml medium. Cultures were incubated in air at 37°C in a rotary shaker with an agitation speed of 160 r/min.

1.3.2 Immobilized cultures

In immobilized culture, 1.8 g carriers were added in 250-ml Erlenmeyer flask containing 100 ml medium. After addition, the carriers were in critical immerged status. Comparably, 0.9 g carriers were added in flask containing 50 ml medium. The inoculum size was the same as that adopted in free culture.

1.4 Analytical methods

Lignin peroxidase (LiP) activity was measured as described by Tien and Kirk (1988), with one unit defined as 1 μmol veratryl alcohol oxidized to veratraldehyde per minute.

Manganese peroxidase (MnP) activity was measured spectrophotometrically by the method of Paszczynski *et al*. (1988), using Mn^{2+} as the substrate. One unit was defined as the amount of enzyme that oxidized 1 μmol Mn^{2+} per minute.

Protease activity was measured with azocoll (Sigma Chemical Co., USA) as the substrate in 50 mmol/L acetate buffer, as described by Dosoretz *et al*. (1990b).

Nitrogen ammonium content was determined by the phenol-hypochlorite method at 625 nm as described by Weatherburn (1967), using ammonium sulfate as a standard.

Reducing sugars were determined by dinitrosalicylic acid method at 540 nm as described by Ghose (1987), using D-glucose as a standard.

2 Results

2.1 Free cultures

2.1.1 Cultured with different volumes of N-limited medium

In the 250-ml Erlenmeyer flasks with 50 or 100 ml nitrogen-limited medium, hyphal pellets were formed since the day 2. After the pellets grew up to about 5 mm in diameter, spurs began to appear and MnP activity emerged in the medium ever since.

Different volumes of medium in flasks led to different nutrition consumption rates (Fig.1). On one hand, ammonium nitrogen was totally consumed in 2 d when 50 ml medium was added, but it was depleted on day 3 in cultures with 100 ml medium. On the other hand, faster average glucose consumption rate was found in 50 ml-medium cultures, which are 0.407 and 0.845 g/(L·d) during the primary metabolism phase (0–4 d) and secondary metabolism phase (5–9 d). In 100 ml-medium culture, glucose consumption rates are 0.337 and 0.810 g/(L·d) during these two phases.

In these two cultures, protease activity curves were approximately the same with maximum value of around 2.4 U/ml on the day 6. However, the time when MnP arrived its maximum and its peak value were quite different. Fermented with 50 ml medium, MnP activity was detected since the day 3 and peaked on the day 4 with a value of 231 U/L. It sharply decreased after that. While in culture containing 100 ml medium, MnP appeared since the day 4 and reached its maximum value of 240 U/L on the day 7. Very few LiP activities were detected in these two culture systems (less than 2 U/L).

In both cultures, after the peak MnP activity the medium became more and more viscous because of the secretion of extracellular polysaccharide and the hyphal pellets began to disaggregate.

2.1.2 Cultured with different volumes of C-limited medium

In C-limited medium free cultures, hyphal pellets also formed since the day 2, but they persisted for a longer time compared to those in N-limited medium free culture mentioned above. Ammonium nitrogen all reached its minimum on the day 4 and kept stable thereafter, but glucose was depleted more rapidly when 50 ml medium was employed (Fig.2).

In spite of different volumes added in the flasks, protease

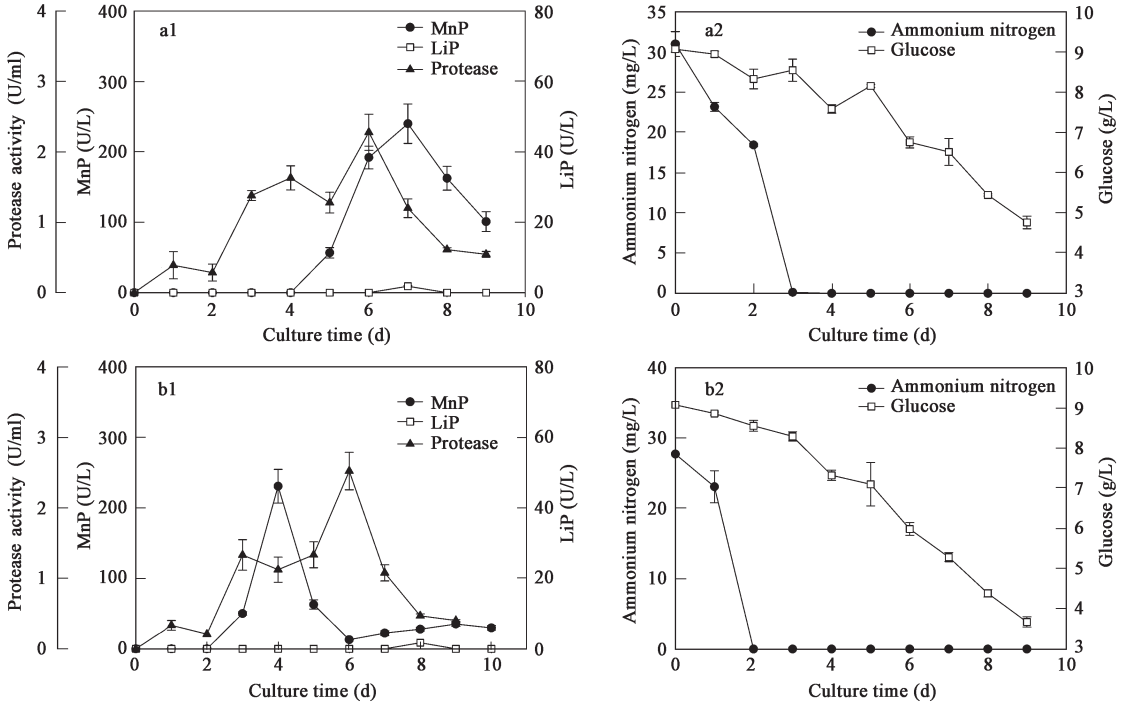

Fig. 1 Glucose, ammonium nitrogen concentration and MnP, LiP, protease activity curves during the free culture with N-limited medium (C/N ratio is 56/2.2, in mmol/L). In 250-ml Erlenmeyer flask, 100 ml (a) and 50 ml (b) medium were added, respectively.

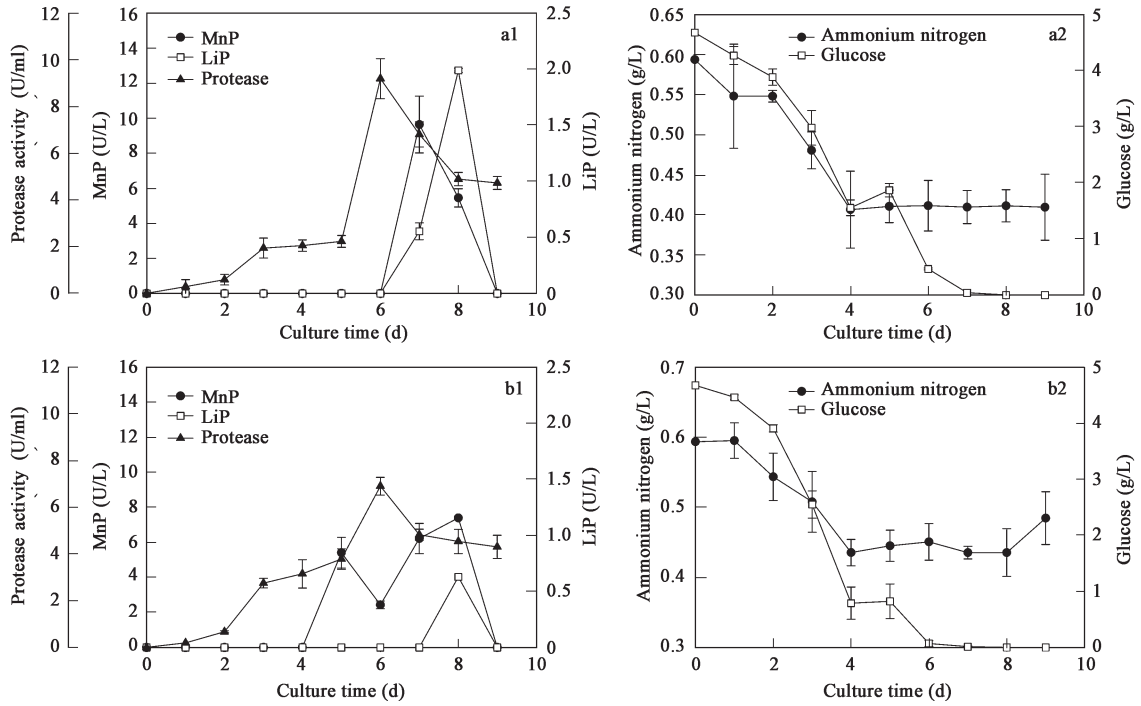

Fig. 2 Glucose, ammonium nitrogen concentration and MnP, LiP, protease activity curves during the free culture with C-limited medium (C/N ratio is 28/44, in mmol/L). In 250-ml Erlenmeyer flask, 100 ml (a) and 50 ml (b) medium were added, respectively.

activity curves showed the same trend during the culture. When *P. chrysosporium* entered the secondary metabolism period, protease activity began to increase (Fig.2), which finally arrived maximum (10 U/ml for 100 ml medium culture and 7 U/ml for 50 ml medium culture) when glucose concentration in the culture broth was low (less than 1 g/L).

MnP and LiP activities were low no matter 50 ml or 100 ml medium was added in culture. MnP activity was less than 10 U/L and LiP was less than 2 U/L.

2.2 Immobilized cultures

2.2.1 Cultured with different volumes of N-limited medium

Since the immersion status of the support in the medium had effect on ligninolytic enzymes production (Yu *et al.*,

2005), in 50 and 100 ml nitrogen-limited medium, 0.9 and 1.8 g polyurethane foam carriers were added, respectively. This made both of these two culture systems in critical immersed conditions.

Fewer mediums in the flask promoted ammonium nitrogen and glucose consumption by fungus. In 50 ml-medium system ammonium nitrogen was depleted on the day 1 while it cost 2 d for complete nitrogen consumption in 100 ml-medium system. Glucose disappeared at a rate of 1.372 g/(L·d) during the whole fermentation process in 50 ml-medium flask, which is faster than the rate of 0.911 g/(L·d) in 100 ml-medium flask (Fig.3). Comparing results got in free and immobilized cultures, we can see that immobilization greatly improved nutrition take-in speed.

Difference was also found in MnP, LiP and protease activity in these two systems. Maximum MnP activity of 410 U/L was reached on the day 5 in flasks containing 50 ml medium, while in 100 ml-medium culture, 321 U/L MnP was produced on the day 10. MnP in the latter system was more stable compared to former MnP activity, although its peak value was less. The highest LiP activity was attained in culture system with 50 ml medium. On the day 5, it reached 721 U/L. However, the other system did not produce LiP at all. Protease activity curve in 50 ml-medium system showed 2 peaks (1.43 U/ml on the day 2, and 0.8 U/ml on the day 8), which were nominated primary protease peak and secondary protease peak in time sequence (Rothschild *et al*., 1999). Actually it was very low compared to that in 100 ml-medium system. In the 100 ml medium system, protease activity was higher than 3 U/ml since the day 3.

The viscosity of the culture broth gradually increased with the culture age for extracellular polysaccharide production as happened in free culture.

2.2.2 Cultured with different volumes of C-limited medium

In the immobilized culture, carbon-limited medium was also employed to replace nitrogen-limited medium. As an effect of immobilization, nutrition disappeared quickly in the medium. Glucose was depleted on the day 2 and ammonium nitrogen reached its minimum at the same time in both systems (Fig.4).

Under these culture conditions, ligninolytic enzymes productions were comparatively low. In 50 ml-medium flasks the maximum MnP activity of 33 U/L was achieved and maximum LiP was 37 U/L. Less MnP and LiP were produced in 100 ml-medium culture, their maximum was 22 U/L and 3.1 U/L, respectively (Fig.4).

With carbon-limited medium, the culture broth kept clear from the beginning till the end of the culture.

3 Discussion

3.1 Ligninolytic enzymes production

To promote ligninolytic enzymes production in large scale, harvesting these products during fermentation in air is undoubtedly of great significance. MnP formation was generally less affected by oxygen level (Rothschild *et al*., 1999) and its production can be easily achieved in flasks or reactors with commonly used nitrogen-limited medium when exposed to air (Couto *et al*., 2001). This viewpoint is also demonstrated in the present research because of high MnP activity in all cultures with nitrogen-limited medium. However, LiP was seldom produced with the same medium without pure oxygen exposure (Rothschild *et al*., 1999; Couto *et al*., 2002b). In this report, the influences of medium type, culture type and medium volume

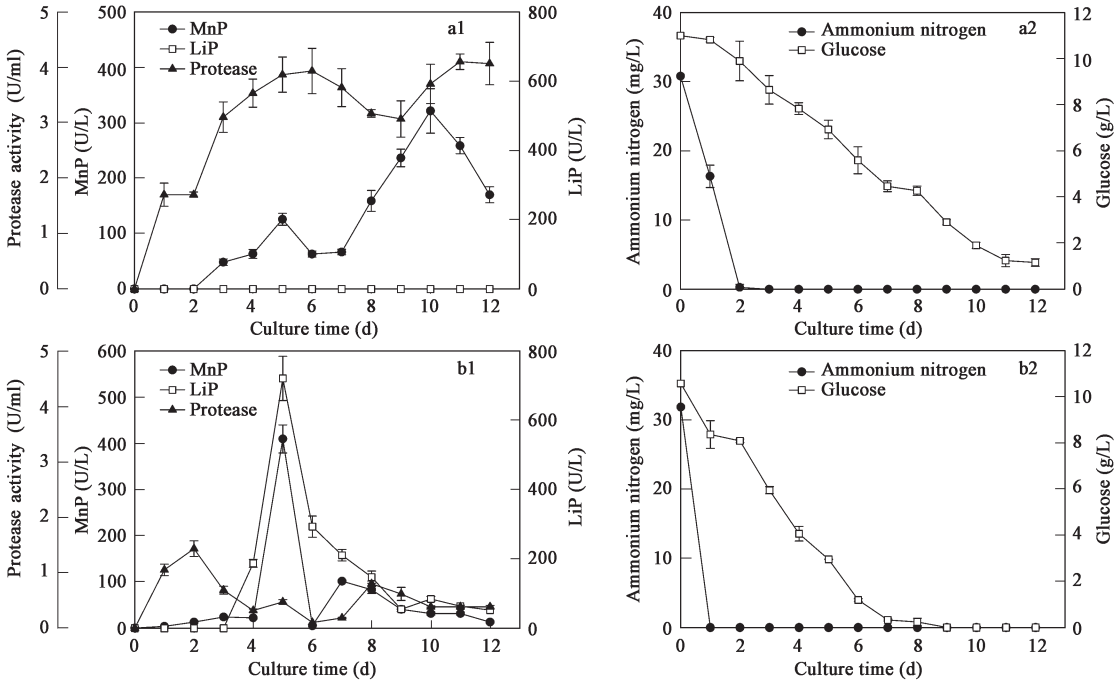

Fig. 3 Glucose, ammonium nitrogen concentration and MnP, LiP, protease activity curves during the immobilized culture with N-limited medium (C/N ratio is 56/2.2, in mmol/L). In 250-ml Erlenmeyer flask; (a) 100 ml medium and 1.8 g polyurethane carriers; (b) 50 ml medium and 0.9 g polyurethane carriers were added, respectively.

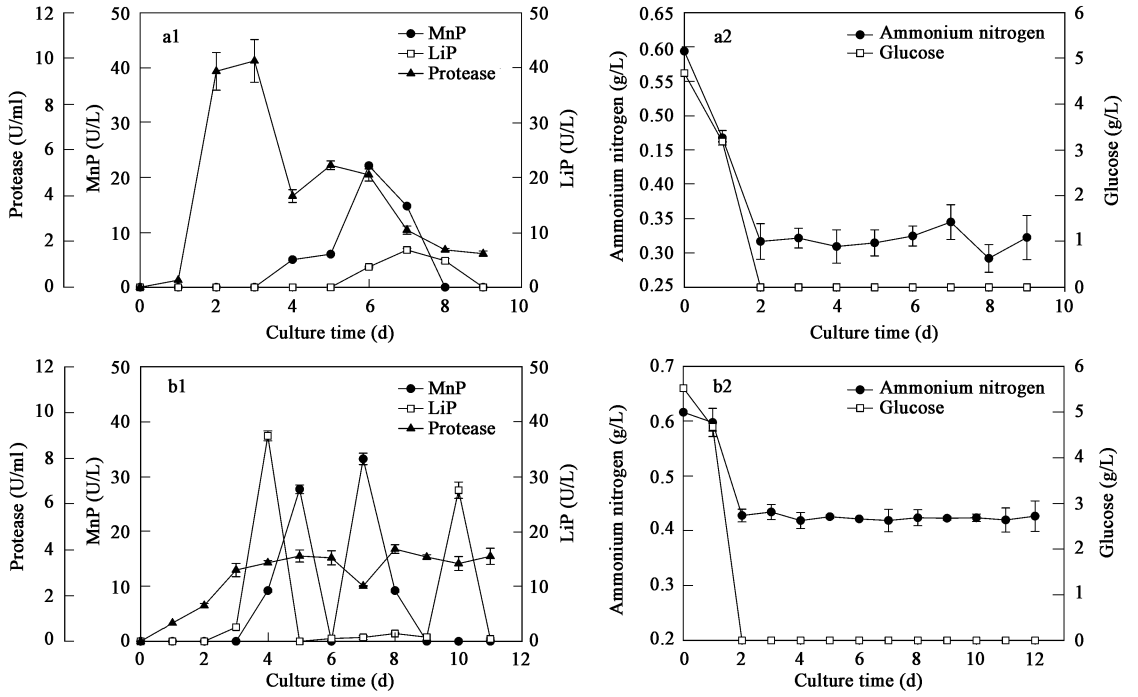

Fig. 4 Glucose, ammonium nitrogen concentration and MnP, LiP, protease activity curves during the immobilized culture with C-limited medium (C/N ratio is 28/44, in mmol/L). In 250-ml Erlenmeyer flask, (a)100 ml medium and 1.8 g polyurethane carriers, (b) 50 ml medium and 0.9 g polyurethane carriers were added, respectively.

on ligninolytic production were studied. Meanwhile, by employing shallow immobilized culture we synchronously got high level of MnP and LiP production in air with nitrogen-limited medium.

When different amounts of medium were employed in cultures, the transfer of oxygen from the air to biomass could be influenced (Couto *et al*., 2000). As the oxygen transfer speed may be the main limitation for fungus growth in liquid culture, the metabolite including ligninolytic enzymes and its deactivators could be released at different time. In our result, the free culture with 50 ml nitrogen-limited medium gave maximum MnP activity earlier and MnP activity decreased more quickly (Fig.1). Zhang (1999) reported similar phenomenon for LiP when 50 and 90 ml medium were employed separately in 250-ml flasks.

In immobilized culture, the polyurethane foam helped the fungus stretch out its hypha. This made it easier to assimilate nutrition and secret metabolites compared to the structure of fungal pellets. Actually, ammonium nitrogen and glucose consumption rates were higher when fungus hyphal was immobilized under all culture conditions in our experiment. Higher MnP activity was attained with the favor of carriers in both 50 and 100 ml nitrogen-limited medium. What is more, much LiP was produced in immobilized 50 ml-medium culture (Fig.3). On the other hand, immobilization can effectively reduce the shear stress which is reported to have inverse effect on LiP production and stability. The only high LiP production in immobilized 50 ml-medium culture reconfirmed the crucial importance of shear stress control and oxygen supply during LiP production process. The results also implied that culture under shallow immobilized conditions was a possible way to gain high activity of ligninolytic enzymes in air.

Nitrogen-limited medium seems to be a better substrate because in these cultures higher ligninolytic enzymes production was achieved. However, because of the excessive glucose, production of polysaccharide during the late fermentation period made the broth viscous (Rothschild *et al*., 1999). It could hamper the diffusion of oxygen and other nutrient, production and secretion of ligninolytic enzymes would be inhibited in succession. In order to make ligninolytic enzymes production more stable or realize its accumulation in nitrogen-limited culture, measures should be taken to avoid the influence of polysaccharide.

3.2 Protease production

Since protease is a factor which may influence ligninolytic enzymes stability during the culture of *P. chrysosporium*, studying the effect of culture conditions on protease production is helpful to direct fermentation process aiming at stable enzymes production.

Of all the three factors involved in this research, medium type has the greatest effect on protease production. In all culture conditions when medium type was the only difference, protease production was always higher in C-limited system than that in N-limited system. The highest protease activity was achieved in immobilized culture with 100 ml C-limited medium in flask. In addition, protease curves were about the same with the same medium in free culture. This result implies *P. chrysosporium* secreted more protease in response to glucose starvation although both glucose and ammonium limitation could stimulate protease secretion.

Another common feature for C-limited system is that the

protease maximum appeared when glucose was completely consumed and all protease curves have no obvious peaks for primary and secondary protease. So far as we know, there is no report about protease production when cultured with C-limited medium. In free culture with N-limited medium and pure oxygen flushing, Dosoretz *et al*. (1990b) tried three kinds of initial glucose concentration. Their results showed that when glucose was depleted protease concentration increased, which was regarded as secondary protease. However, Dass *et al*. (1995) employed a medium containing excessive nitrogen source in their research and they suggested protease produced during the whole process was primary protease. So, we cannot determine which type the protease secreted in C-limited system belongs.

The culture type and medium volume might act corporately because they both can influence oxygen supply to biomass which was reported to be a factor for protease secretion (Dosoretz *et al*., 1990b; Zhen and Yu, 1998). Immobilization favored biomass growth, which also increased nutrition, including glucose, ammonium nitrogen and oxygen consumption rate, as well as oxygen demand. Fewer medium in flask means more effective oxygen transfer with the same shaking speed. In immobilized culture, more oxygen is needed and 100 ml medium system cannot meet this requirement. Fungus was in status of oxygen starvation (measured DO concentration was about 0.2 mg/L) and subsequently secreted more protease (Figs.3 and 4). Less difference in free cultures with 50 ml medium and 100 ml medium was found because less oxygen was needed (Figs.1 and 2).

The protease in immobilized culture with 50 ml N-limited medium had the lowest activity (1.4 U/ml) in our experiment, which is also the lowest in all the reports we have found. Dosoretz *et al*. (1990b) measured the primary and secondary protease maximum activity in submerged liquid culture, which was about 6 U/ml. This could have been caused by pure oxygen flushing as increased oxygenation simultaneously increased protease activity (Dosoretz *et al*., 1990b). This also can explain why protease was above 20 U/ml in solid-state culture (Cabaleiro *et al*., 2002). Another comparatively low protease activity (3.5 U/ml) was observed in free culture of *P. chrysosporium* under air atmosphere. These results indicate that protease production can be minimized with proper culture conditions in air.

3.3 Relationship between protease and ligninolytic enzymes

Although the culture conditions affected both ligninolytic enzymes and protease production, relationship between protease and ligninolytic enzymes was discussed in many research reports.

Protease was firstly found to cause LiP degradation by Dosoretz *et al*. (1990b, c) and secondary protease was used in his experiment. Another research proved that the primary protease could totally denatured LiP (Pascal *et al*., 1993). In our result, high LiP production was only realized in the immobilized culture with 50 ml N-limited medium, where the lowest primary and secondary protease activity was detected. This indicated that protease produced during the culture is an important factor which reduces LiP production and it needs to be regulated to achieve higher LiP production in *P. chrysosporium* fermentation. It is reported that protease could be inhibited with addition of substances, such as PMSF (phenyl methane-sulfongl fluoride) and glucose (Dosoretz *et al*., 1990b) and consequently ligninolytic enzymes could be reproduced in the culture (Yu *et al*., 2005). Our experimental results proved that protease secretion could be controlled by adopting proper culture conditions, which subsequently led to high LiP production.

The relationship between protease and MnP is more complicated. Cabaleiro *et al*. (2001) presented the viewpoint that MnP activity is inhibited by extracellular protease with the fact that MnP was more stable when protease activity was inhibited. However, recently another viewpoint came up that the first peak of extracellular protease helped MnP secretion by participating in hyphal autolysis steps, while the protease produced during late idiophase would play a role in the decline of MnP (Jimenez *et al*., 2003). Although the MnP and protease were reversely correlated in some cultures (e.g. Fig.1b), MnP still increased when protease activity was high (e.g. Fig.1a). It implies that protease isn't a main factor for MnP production and stability.

Acknowledgements

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