

Influence of Carbon Source on the Production of Extracellular Ligninolytic Enzymes by *Phanerochaete chrysosporium*

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The effect of altering the carbon source in the growing environment was investigated relative to the production of ligninolytic enzymes by *Phanerochaete chrysosporium*. Glucose, cellobiose, and cellulose (or mixtures thereof) were used as the carbon sources. Glucose oxidase and glyoxal oxidase activities in all carbon sources were produced during cultivation. High peak levels (0.17 to 0.24 IU/mL) of manganese peroxidase activity were observed only in mediums containing oligosaccharides. Lignin peroxidase activity was high in glucose medium (0.21 IU/mL of peak value); however, minimal amounts were formed in the cellulose medium (0.01 IU/mL of peak value). High amounts of cellobiose:quinone oxidoreductase (3.33-3.99 IU/mL of peak value) and cellobiose dehydrogenase (0.04-0.2 IU/mL of peak value) were measured when *P. chrysosporium* was grown on a medium containing cellulose. This work discovered that the mixture of glucose and cellulose as a carbon source favored high co-production of ligninolytic enzymes by *P. chrysosporium*.

Keywords: Carbon source; Ligninolytic enzyme; *Phanerochaete chrysosporium*; Lignin degradation

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INTRODUCTION

Lignin, a major component associated with cellulose microfibrils in plant cell walls, is the second most abundant natural polymer and the most abundant aromatic material on earth. This amorphous and insoluble aromatic material lacks the stereoregularity present in cellulose. Lignin's structural features demand its initial biodegradative systems to be extracellular, nonspecific, and nonhydrolytic (Kirk and Farrell 1987). These features are identified as major obstacles to the enzymatic hydrolysis of lignocellulose biomass (Ding *et al.* 2012) and its high-value use (Bruijninx and Weckhuysen 2014). Lignin depolymerization is an important starting point for many lignin valorization strategies (Ragauskas *et al.* 2014). In nature, white rot fungi are the only known organisms that completely break down lignin to CO₂ and H₂O, thereby gaining access to the carbohydrate polymers in the plant cell walls, which are used as carbon and energy sources (Martinez *et al.* 2004). White rot fungi secrete extracellular ligninolytic enzymes that combine with other processes to affect lignin mineralization (Blanchette *et al.* 1997). The extracellular ligninolytic enzymes have the high non-specificity to the substrate range (Pizzul *et al.* 2009). A fortuitous result of this is that ligninolytic enzymes of white rot fungi have been demonstrated to be capable of transformation or mineralization of a wide range of highly

recalcitrant organic pollutants with structural similarities to lignin (e.g. polycyclic aromatic hydrocarbons, pesticides, organic chlorines, polychlorinated biphenyls) (Pointing 2001). Therefore, white rot fungi are one of the main bioremediation microbe classes (Chen *et al.* 2015). Lignin biodegradation by white rot fungi has important significance to the lignin high-value utilization, pulp and paper industry, bioremediation, and biorefinery. Of the white rot fungi, *Phanerochaete chrysosporium* is the most intensively studied.

P. chrysosporium catalyzes the depolymerization of lignin by an array of extracellular oxidases, peroxidases, and dehydrogenases. Among these enzymes, lignin peroxidase (LiP) and manganese peroxidase (MnP) activate lignin to generate highly reactive and nonspecific free radicals, which in turn undergo a complex series of spontaneous cleavage reactions (Higuchi 2004; Bugg *et al.* 2011). Glucose oxidase and glyoxal oxidase (GLOX) provide H₂O₂ for peroxidases (Kelley and Reddy 1986a; Kersten and Kirk 1987). Cellobiose:quinone oxidoreductase (CBQ) and cellobiose dehydrogenase (CDH) can reduce aromatic radicals formed by ligninolytic enzymes in the presence of cellobiose (Samejima and Eriksson 1991; Bao *et al.* 1993), thereby preventing the repolymerization of aromatic radicals and supporting lignin degradation. Accordingly, the co-production of these enzymes in *P. chrysosporium* is critical for lignin degradation.

LiP, MnP, glucose oxidase, and GLOX are produced from *P. chrysosporium* grown in medium with 10 g/L or 20 g/L of glucose (Tien and Kirk 1984; Glenn and Gold 1985; Kelley and Reddy 1986b; Kersten 1990). CBQ and CDH are produced when cellobiose or cellulose is the carbon source (Henriksson *et al.* 2000). Using cellulose as the carbon source, high titres of LiP are produced in submerged liquid cultures with continuous agitation in air, but neither MnP, laccase, nor CDH activity is detected (Zacchi *et al.* 2000). Kapich *et al.* (2004) reported that lignocellulose medium simultaneously produces high activities of both MnP and LiP in submerged fungal cultures. Singh *et al.* (2011) showed that adding 2% mannose to the medium stimulated the best MnP production. However, there is a considerable lack of reports regarding the effect of carbon sources on glucose oxidase and GLOX. And the effect of carbon source on co-production of those ligninolytic enzymes from *P. chrysosporium* has not been reported so far.

This study investigated the influence of glucose, cellobiose, and cellulose as carbon sources on the co-production of lignin-degrading enzymes. *P. chrysosporium* ME 446 was grown on different substrates containing these carbon sources, either alone or in combinations, and the ligninolytic enzyme production was investigated.

EXPERIMENTAL

Reagents

Veratryl alcohol, 2,6-dimethoxyphenol (2,6-DMP), 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid, denoted ABTS), and horseradish peroxidase (HRP) were purchased from Sigma (Shanghai, China). 2,2-dimethylsuccinic acid was purchased from Tianjin Heowns Biochemical Technology Co., Ltd (Tianjin, China). Other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

Fungal Strain and Culture Condition

Phanerochaete chrysosporium ME 446 was maintained on potato dextrose agar (PDA) slants at 4 °C and precultured on PDA plates at 37 °C. The inoculum consisted of conidial suspensions ($A_{650nm} = 0.65/cm$) that were filtered through white glass wool. The

conidia were washed away from 3- to 4-week-old cultures grown on PDA plates using sterile double-distilled water. The growth medium was prepared as previously reported, accounting for variations in the type of carbon source and the use of 3 mM veratryl alcohol (Tien and Kirk 1988). Six kinds of carbon sources were used. The carbon sources containing one kind of carbohydrate were glucose (1%, w/v), cellobiose (1%), or cellulose (1%). The other three kinds of mixture carbon sources were glucose (0.5%) plus cellobiose (0.5%), glucose (0.5%) plus cellulose (0.5%), and cellobiose (0.5%) plus cellulose (0.5%), respectively. There were 10 mL of inoculums per 100 mL of culture. Erlenmeyer flasks (100-mL) containing 10 mL of culture were cultivated at 37 °C at 80% relative humidity in an incubator.

Enzyme Activity Assay

The activities of LiP, MnP, CBQ, CDH, glucose oxidase, and GLOX were measured in the extracellular medium after the fungal mycelia was filtered. For the LiP, CBQ, CDH, glucose oxidase, and GLOX controls, the extracellular culture liquid was boiled for 10 min, and the reaction mixture lacking Mn^{2+} served as a control for MnP activity. One unit of LiP, MnP, CBQ, and CDH enzyme activity was defined as the amount of enzyme required to form 1 μ mol of reaction product per min, which was expressed as IU/mL (or IU/L). LiP activity was measured according to Arora and Gill (2001), using veratryl alcohol as the substrate. For MnP, the oxidation of 2,6-DMP was used to measure enzyme activity (Field *et al.* 1992). CBQ and CDH activity were measured according to Samejima and Eriksson (1992). Glucose oxidase and GLOX activities were measured according to Urzúa *et al.* (1998), with the following modifications. A 0.375-mL sample was combined with 0.25 mL of glucose (100 mM) or formaldehyde (100 mM) and 0.125 mL of 100 mM 2,2-dimethylsuccinate sodium (pH 4.5). The resulting mixtures were incubated at 30 °C for 30 min. The produced assay of H_2O_2 was the same as the H_2O_2 standard curve as follows, accounting for 0.1 mL of reaction liquid substituted for 0.1 mL H_2O_2 . The H_2O_2 standard curve was prepared as follows: The concentration of 100 μ M H_2O_2 was prepared with 30% H_2O_2 . The accurate content of H_2O_2 in 30% H_2O_2 solution had been determined by the calibrated sodium hyposulfite standard solution beforehand. The reaction mixture (total volume, 0.5 mL) contained 0.1 mL of H_2O_2 (0, 10, 20, 40, 60, 80, or 100 μ M), 0.4 mL ABTS (10 mM), and 6 IU HRP in 50 mM potassium phosphate buffer (pH 6.5). The assay was conducted at 30 °C for 15 min and terminated by adding 0.3 mL of 5 M HCl. The absorbance was measured at 420 nm. The linear correlation between the absorbance and the H_2O_2 concentration was used for calculating the activity of glucose oxidase and GLOX.

Data Analysis

All results were calculated as the mean \pm standard error (SE) of three replicates. The linear regressions were performed with Origin 9.0 software (OriginLab Corporation, Northampton, USA).

RESULTS AND DISCUSSION

Influence of Carbon Source on LiP Production

Figure 1 charts the LiP activity from *P. chrysosporium* grown in the six types of carbon sources. The LiP activity of *P. chrysosporium* in both the glucose and cellobiose

cultures reached their peak values of 0.211 IU/mL and 0.062 IU/mL, respectively, at day 12. Low levels of LiP activity were present in the cellulose cultures throughout cultivation (Fig. 1). The LiP activity of *P. chrysosporium* growing in a glucose-cellobiose medium reached a maximum of 0.093 IU/mL after 12 d. Peak levels (0.091 IU/mL) of LiP activity from *P. chrysosporium* growing in a glucose-cellulose medium were attained on day 6. The highest value (0.054 IU/mL at day 10) of LiP activity in cellobiose-cellulose medium were obtained 4 d later than in the glucose-cellulose medium (Fig. 1). In a descending order of the peak values of LiP, the carbon sources were in the sequence glucose, glucose-cellobiose, glucose-cellulose, cellobiose, cellobiose-cellulose, and cellulose. These findings indicated that glucose was beneficial to the production of LiP, whereas cellobiose and cellulose were not used by *P. chrysosporium* until they were hydrolyzed by extracellular hydrolase that the fungus secreted, so the LiP production was less. It can be explained that the glycan hydrolase that *P. chrysosporium* secreted inhibited the production of LiP. Its mode of action may be the hydrolysis of the glycone moiety of LiP by extracellular glycan hydrolase, which decreased the activity of LiP (Berg-Fussman *et al.* 1993). Or some factors produced in the expression of the extracellular glycan hydrolase inhibited the expression of LiP.

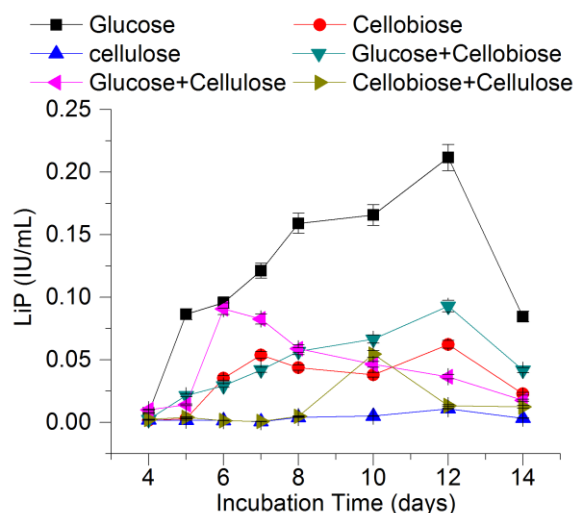


Fig. 1. Time course of LiP activities from *P. chrysosporium* in six kinds of carbon source cultures

Influence of Carbon Source on MnP Production

The time course of MnP activity is shown in Fig. 2. *P. chrysosporium* in glucose medium reached its maximum MnP value (0.176 IU/mL) on day 7, after which it gradually decreased. A similar trend was observed for the cellobiose, glucose-cellobiose, and glucose-cellulose media (Fig. 2). The two peak values of MnP activity from *P. chrysosporium* growing in the cellobiose-cellulose medium were found on day 6 (0.078 IU/mL) and day 10 (0.067 IU/mL). In a similar manner, there were two peak values of MnP activity in the cellulose medium, where peaks were found on day 8 (0.034 IU/mL) and day 12 (0.050 IU/mL). The two peaks in cellobiose-cellulose and cellulose media were due to the slow release of the directly-available glucose after cellulose hydrolysis. In a descending order of the peak values of MnP, the carbon sources were in sequence glucose-cellobiose, cellobiose, glucose, glucose-cellulose, cellobiose-cellulose, and cellulose. These findings indicated that the glucose or cellobiose media were favorable for the production of MnP. In agreement with the production of LiP, the cellulose media was not advantageous to the production of MnP.

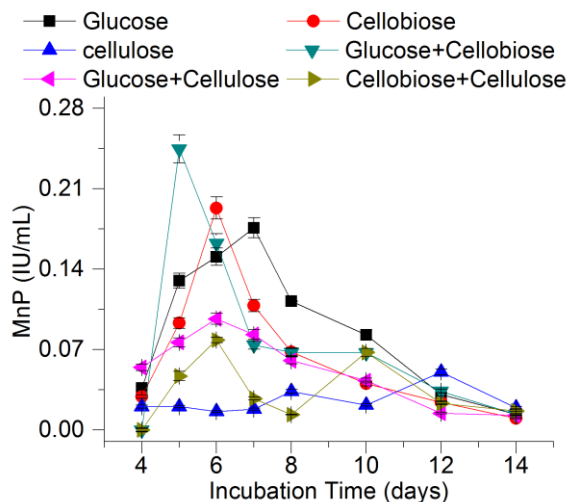


Fig. 2. Time course of MnP activities from *P. chrysosporium* in six kinds of carbon source cultures

Influence of Carbon Source on CBQ and CDH Production

The formation of extracellular CBQ (Fig. 3A) and CDH (Fig. 3B) by *P. chrysosporium* grown in the same carbon source exhibited similar trends. The amounts of extracellular CBQ and CDH formed in the glucose medium from day 5 to day 14 were negligible.

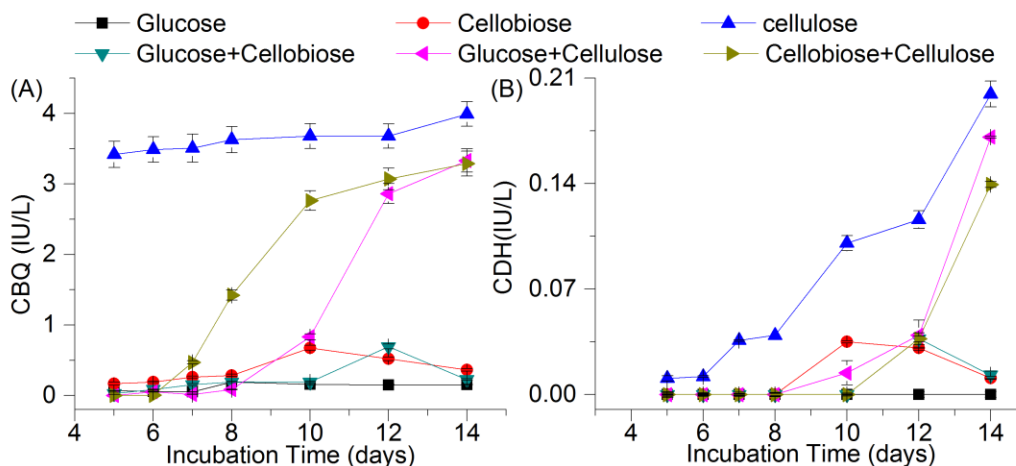


Fig. 3. Time course of CBQ (A) and CDH (B) activities from *P. chrysosporium* in six kinds of carbon source cultures

In the cellobiose medium, CBQ and CDH activity reached the maximum of 0.68 IU/L and 0.035 IU/L, respectively, at day 10. Similarly, the highest CBQ and CDH activity in the glucose-cellobiose medium were 0.69 IU/L and 0.037 IU/L, respectively, at day 12.

CBQ activity in both the glucose-cellulose and cellobiose-cellulose media gradually increased from days 5 to 14. CDH activity in the glucose-cellulose and cellobiose-cellulose media was not initially detected, but it increased to its maximum (0.17 IU/L and 0.14 IU/L, respectively) on day 14. However, in comparison with CBQ, CDH activity in the glucose-cellulose medium was higher than the cellobiose-cellulose medium.

For the cellulose medium, CBQ and CDH activity gradually increased during cultivation, and their peak values were 3.99 IU/L and 0.2 IU/L, respectively. As previously reported (Henriksson *et al.* 2000), the media containing cellulose was favorable for CBQ and CDH production, the media that contained cellobiose but not cellulose only produced low CBQ and CDH activities, and the media in which glucose was carbon source did not sustain the production of CBQ and CDH.

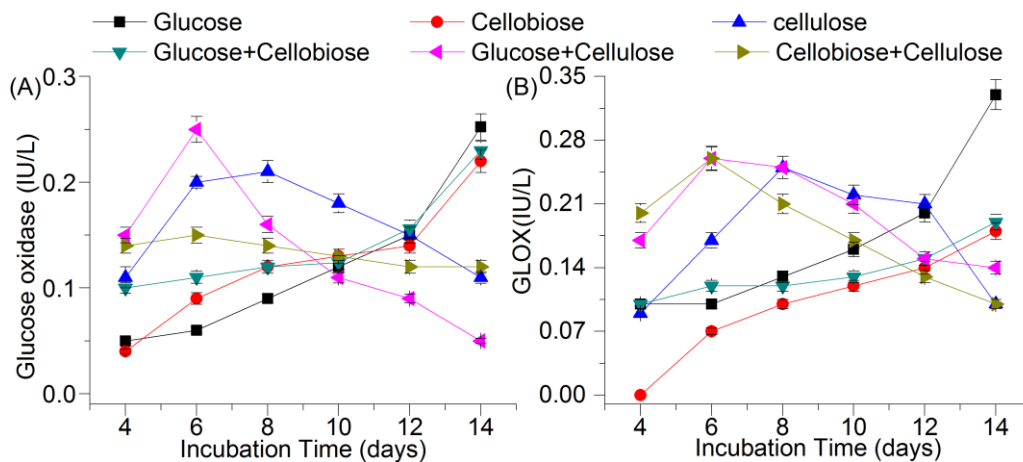


Fig. 4. Time course of glucose oxidase (A) and GLOX (B) activities from *P. chrysosporium* in six kinds of carbon source cultures

Influence of Carbon Source on Glucose Oxidase and GLOX Production

Assays for glucose oxidase activity (Fig. 4A) and GLOX activity (Fig. 4B) yielded similar results. In the glucose medium, the overall production of glucose oxidase and GLOX by *P. chrysosporium* progressively increased, and the peak activity (0.25 IU/L and 0.33 IU/L, respectively) was detected on day 14.

A similar production pattern occurred when *P. chrysosporium* was grown on a cellobiose medium, although the peak values of glucose oxidase (0.22 IU/L) and GLOX activity (0.18 IU/L) were slightly lower. For the glucose-cellobiose medium, enzyme activity also increased, but the increase was slower than when either glucose or cellobiose was the sole carbon source.

Glucose oxidase and GLOX activity were different in the medium containing cellulose (Fig. 4). Glucose oxidase and GLOX activity reached their maximum on day 6 in the oligosaccharide-cellulose medium. The peak levels of glucose oxidase and GLOX activity were 0.25 IU/L and 0.26 IU/L, respectively, in the glucose-cellulose medium. For the cellobiose-cellulose medium, the maximum glucose oxidase and GLOX activity corresponded to 0.15 IU/L and 0.26 IU/L, respectively. For the cellulose medium, the peak level of glucose oxidase (0.21 IU/L) and GLOX (0.25 IU/L) activity were attained at 48 h, following the highest level of activity for the oligosaccharide-cellulose medium.

The maximum LiP, MnP, CDH, glucose oxidase, and GLOX activity from *P. chrysosporium* in different media are depicted on a radar graph (Fig. 5). The graph excludes CBQ, as the peak level of CBQ activity was ten times higher than the other enzyme activities. Nevertheless, its response was not ignored because the peak value pattern for the CBQ activity corresponded to the CDH activity in the same carbon source. The results were similar to a previous study of cellulose medium (Costa-Ferreira *et al.* 1994). Other

papers explain that CBQ is a non-heme form of CDH, created by proteolytic cleavage (Henriksson *et al.* 1991; Wood and Wood 1992).

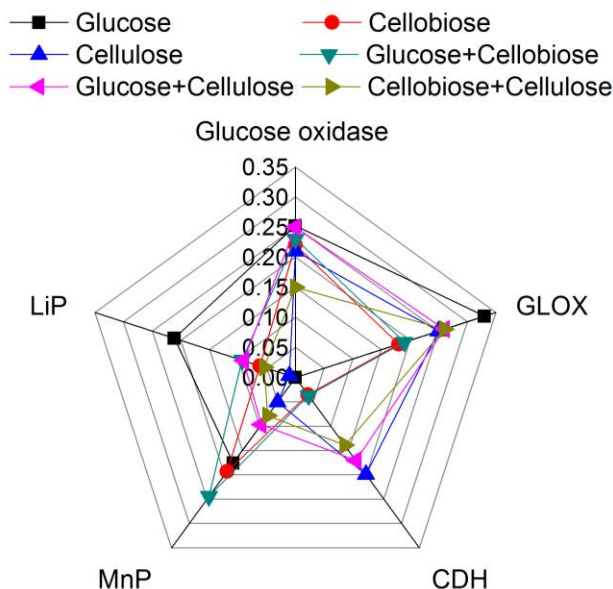


Fig. 5. Radar graphs illustrating the peak levels of ligninolytic enzyme production from *P. chrysosporium* in different carbon sources

P. chrysosporium produced higher peroxidase activity and little CBQ and CDH activity in the glucose medium (Fig. 5). Additionally, the high peak values of glucose oxidase and GLOX activity were observed in glucose medium; however, the peak appeared later than previously reported (Zhao and Janse 1996). The GLOX activity level was similar to the activities of GLOX during the fermentation of rice straw by *P. chrysosporium* in the optimal medium (Bak *et al.* 2009).

In the cellobiose and glucose-cellobiose media, high MnP activity and low GLOX activity were observed (Fig. 5). Unexpectedly, there was low CDH activity in the cellobiose and glucose-cellobiose media, although it was reported that there were high amounts of CDH in fed batch fermentations of *P. chrysosporium*, after adding limiting amounts of cellobiose or mixtures of cellobiose and glucose (Szabó *et al.* 1996).

As previously reported by Eriksson *et al.* (1993), high CBQ and CDH activities were attained in the medium containing cellulose (Fig. 3 and Fig. 5), in which the maximum activity of glucose oxidase and GLOX appeared earlier than in media containing only oligosaccharides. The low levels of LiP and MnP activity in the cellulose medium agreed with previously reported values (Silva *et al.* 1996).

As diagramed in Fig. 6, during lignin degradation by *P. chrysosporium*, glucose oxidase and GLOX oxidize the corresponding substrates with the formation of hydrogen peroxide, which is a prerequisite for the activation of lignin peroxidase and manganese peroxidase (Kelley and Reddy 1986a; Kersten and Kirk 1987). CBQ and CDH prevent repolymerization reactions of phenoxyradical or cational radicals formed by lignin peroxidase, which facilitates lignin degradation. Therefore, the co-production of LiP, MnP, glucose oxidase, GLOX, CBQ, and CDH favors lignin degradation. However, numerous proteins presumed to be involved in natural lignocellulosic biomass transformation and degradation were expressed and produced in variable quantities in response to different

carbon source (Adav *et al.* 2012). Because lignin biodegradation by white rot fungi contributed to the high-value utilization of lignin, the elimination of enzymatic hydrolysis obstacle of lignocellulose biomass, the decrease of pollution in pulp and paper industry, the remediation of the pollution that was caused by recalcitrant organic pollutants with structural similarities to lignin. The type of carbon source to aid the high co-production of ligninolytic enzymes urgently needs to be determined.

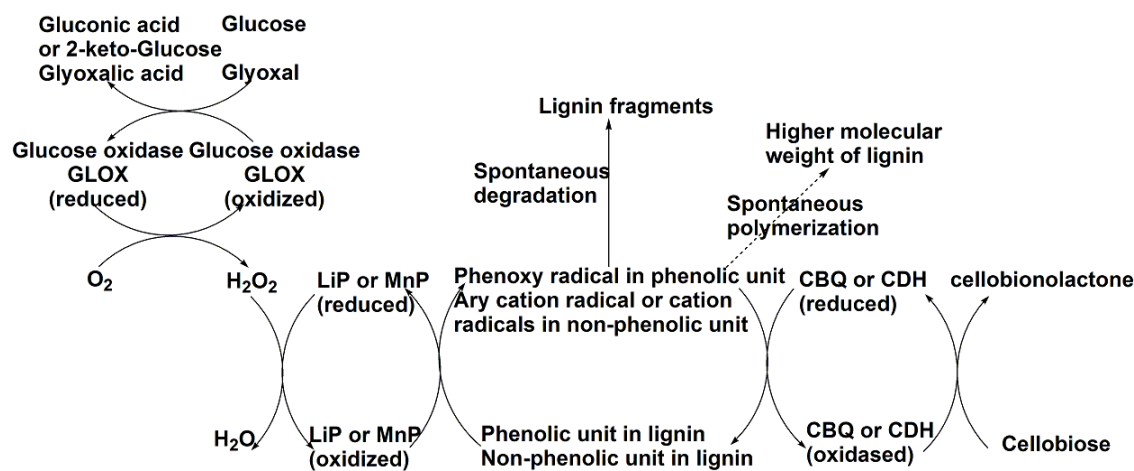


Fig. 6. Schematic graph of lignin degradation by *P. chrysosporium*

In the types of carbon sources that were selected in the study, to achieve high co-production of ligninolytic enzyme by *P. chrysosporium*, a mixture of glucose and cellulose was a better carbon source than others tested in this study (Fig. 5).

CONCLUSIONS

1. The higher peroxidase activity was produced by *P. chrysosporium* grown in a glucose medium, but little CBQ and CDH activity was produced in the same medium. The high peak levels of manganese peroxidase activity were produced in media containing only oligosaccharides. High CDH and CBQ activity was produced in media containing cellulose. Glucose oxidase and GLOX in all the mediums during incubation was secreted by *P. chrysosporium*.
2. The type of carbon source to aid the high co-production of ligninolytic enzyme have been found in *P. chrysosporium*, which produced high levels of extracellular enzymes involved in lignin degradation when the mixture of glucose and cellulose was the carbon source.

ACKNOWLEDGMENTS

The authors are grateful to the National Natural Science Foundation of China for providing financial support (Grant no. 30871985).

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Article submitted: December 30, 2015; Peer review completed: April 9, 2016; Revised version received: April 17, 2016; Accepted: April 24, 2016; Published: May 5, 2016. DOI: 10.15376/biores.11.3.5676-5686