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Cometabolic degradation of veratryl alcohol and biphenyl by white rot fungus under nitrogen nutrition-rich condition *

Lin Lu, Deng Yao-jie, Hu Jian, Zhan Huai-yu

College of Paper and Environment Engineering, South China University of Technology, Guangzhou 510641, China

Abstract—In this paper, changes of enzymes involved in the degradation of recalcitrant aromatic pollutants from white rot fungus *Phlebia radiata* I-5-6 and cometabolism of biphenyl and varatryl alcohol by this fungus under nitrogen nutrition-rich were studied. Results from the experiment showed that C/N concentration ratio in the culture media played an important role on the activity of LiP. Under the condition of high concentration ratio of C/N or N/C, activity of LiP was higher, but higher activity of MnP only followed the low concentration of glucose or tartrate ammonium concentration in the media, when concentration of glucose or tartrate ammonium was more than 0.01 mol/L, the activity of MnP dropped down quickly. Veratryl alcohol increased the activity of LiP and MnP as well as the amount of ·OH radical free under different concentration of nitrogen or carbon source; ascorbic acid, a scavenger of ·OH radical free, brought the opposite effect to that of the veratryl alcohol on the LiP and MnP activity. Under nitrogen nutrition-rich condition removal percentage of biphenyl was lower, however, under cometabolic condition of veratryl alcohol and biphenyl, the degradation was enhanced obviously, moreover, intermediate products accumulated in the media during the cometabolic degradation process of biphenyl and veratryl alcohol was different from these which was found in the process of separate degradation of biphenyl or veratryl alcohol.

Keywords: white rot fungus; cometabolism; degradation.

1 Introduction

White-rot fungi, the ecologically distinct group for their capability of independently degrading lignin component of the plant in the natural environment, are a kind of basidiomycetes. In recent years, there were many reports concentrating on their capability of degrading and mineralizing aromatic pollutants recalcitrant in the environment(Bumpus, 1985; Aust, 1990; Hammel, 1992; Bogan, 1996), and research in the area of using these organisms for bioremediation of the polluted site of soil and waters become the hot attention point in the environment science. To date, studies showed that ligninolytic enzymes of these fungi involved in the degradation process of recalcitrant aromatic pollutants, which are lignin peroxidases (LiP) and manganese-dependent peroxidases (MnP), are only produced under the nitrogen nutrition-limited or carbon nutrition-limited condition, but in our previous studies we found that strain Lu-11 of *Coriolus versicolor* and strain I-5-6 of *Phelebia radiata* could degrade phenolics in the effluent from kraft cooking of bagasse and

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eucalyptus materials and CEH bleaching process of these kraft pulps under nitrogen nutrition-rich or carbon nutrition-rich culture media (Lin, 1996a; 1996b), and also, we found that the degradation of chlorinated guaiacols was greatly enhanced by addition and synchronous degradation of veratryl alcohol in the culture media (Lin, 1996b), the phenomenon of cometabolism was implicated in these results. Since rich nutrition of carbon or nitrogen are often presented in the polluted site of soil and waters, study about degradation of these aromatic pollutants should be very meaningful. In this study, culture media under nitrogen nutrition- rich condition was prepared to investigate the cometabolic degradation of veratryl alcohol and biphenyl by white rot fungus in order to provide the further application basis for in situ bioremediation of aromatic pollution in the environment.

2 Materials and methods

Strain and culture: The strian used in this experiment was *Phlebia radiata* I-5-6 screened in our own laboratory and was inoculated into the culture media, which contained 1.0g/L KH_2PO_4, 0.5g/L $MgSO_4 \cdot 7H_2O$, 0.01g/L $FeSO_4 \cdot 7H_2O$, 0.01g/L $ZnSO_4 \cdot 7H_2O$, 0.01g/L $CaCl_2 \cdot 2H_2O$, 0.001 g/L Vitamin B_1, concentration of 0.01, 0.02, 0.03, 0.04, 0.05 and 0.06 mol/L tartrate ammonium and glucose were respectively added into culture as nitrogen source and carbon source. After 3 days of culture, the mycelial body were taken out to be dispersed to 8×10^6 spores/ml, then inoculated these spores into the culture media of biphenyl (purchased from Aldrich Co.) of concentration 10 mg/L, before culture oxygen was flushed into for 1 minutes, temperature for culture was 37℃. Inorganic ingredients for culture was the same to the above, glucose concentration was 0.01 mol/L, tartrate ammonium was 0.05 mol/L. After 3 days of inoculation time, 0.05 mol/L veratryl alcohol and 0.01 mol/L ascorbic acid was used in the media; after 8 days of inoculation time, liquid were taken from the culture media for ligninolytic enzymes activity assays after filtration with four layers of cotton mesh and then centrifugation (10 min at 400×g).

Lignin peroxidase(LiP) activity was determined by the method of Tien and Kirk(Paszczynski, 1988), and Manganese-dependant peroxidase(MnP) activity was determined as described by Tien (Tien, 1988).

GC-MS assays intermediate product of degradation process of veratryl alcohol and biphenyl was extracted under acidification with 0.1mol/L H_2SO_4 to pH 4.5—5.5 with ethylacetate for three times, then blended and dried in the vacuum. The dried extractives was then dissolved with 1 ml of methanol after desiccation, and went directly into the GC-MS analysis. 1.5μl of methanol solution was injected into Ultra 2 column (50m×0.32mm) with an HP 5972 gas chromatograph, temperature for assays was programmed from 40℃ to 290℃ at 4℃/min, carrier gas was He, pressure for column was 5 psi. For MS analysis the temperature was 150℃, voltage was 70eV with 10—750 mass amu, time of scan period was 2 min, intermediate products was identified by scanning WILEY Chemicals GC-MS Library.

·OH radical free analysis: ·OH radical free measured was according to Backa *et al*. (Backa, 1993). This method was based on the hydroxylation of non-chemiluminescent phthalic hydrazide

(purchased from Aldrich Co.) by hydroxyl radicals in the reaction system to give strongly chemiluminescent 3-hydroxyphthalic acid (3-OHPA) under existence of alkaline H_2O_2 and persulphate. Analysis for chemiluminescence was done under 100 chemiluminator at 560 nm.

3 Results

3.1 Effect of concentration glucose and tartrate ammonium on the activity of LiP and MnP

Different concentrations of glucose and tartrate ammonium had obvious effect on the activity LiP. LiP activity in the culture media was enhanced with increase of concentration of tartrate ammonium when the glucose concentration was fixed at a certain degree, and with increase of glucose at a certain concentration of tartrate ammonium the LiP activity in the culture media also increased(Fig. 1). Therefore, when C/N value was >1 or <1, the activity of LiP was enhanced, the activity of LiP depended on the C/N value, this value tended to 1, the activity decreased, the farther this value left 1, the more the activity increased. However, to the activity of MnP, when carbon or tartrate ammonium concentration was more than 0.01 mol/L, the activity measured in the media decreased all without any exception(Fig.2).

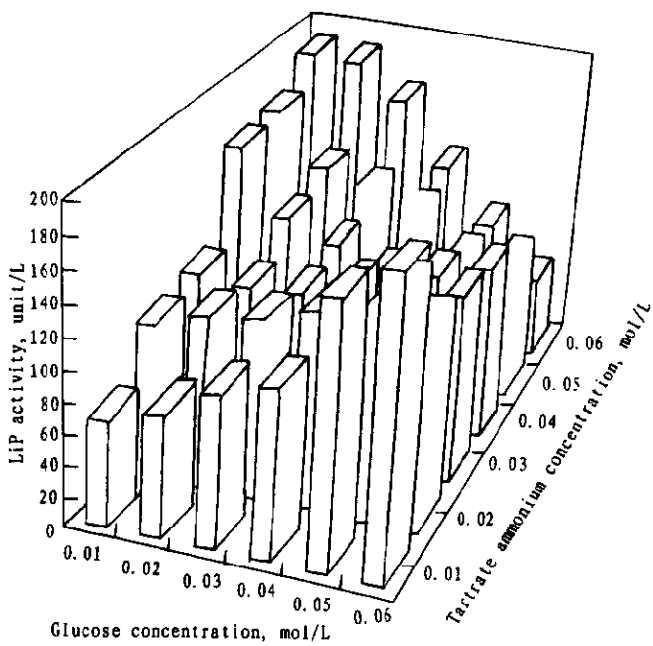

Fig. 1 Effect of glucose and tartrate ammonium concentration on activity of LiP

3.2 Effect of veratryl alcohol and ascorbic acid on the activity of LiP and MnP and amount of ·OH radical free

The activity of LiP and MnP in the culture media in a inoculation period of 8 days rose to the highest respectively at the 6th and 7th day. Veratryl alcohol promoted the activity of LiP and

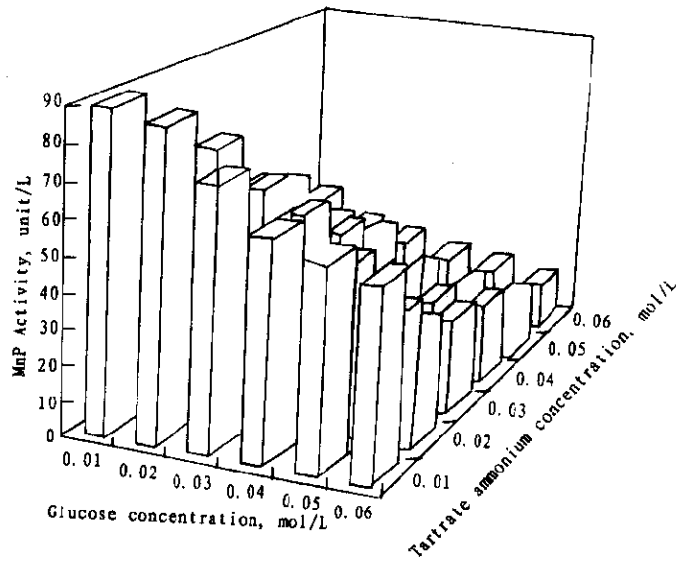

Fig. 2 Effect of glucose and tartrate ammonium concentration on activity of MnP

MnP, but ascorbic acid lowered the activity of LiP and MnP, this result was first observed in our research and was not reported ever before(Fig. 3). For the effect of veratryl alcohol and ascorbic acid on the production of ·OH radical free, it was observed that the amount of ·OH radical free enhanced by veratryl alcohol, but ascorbic acid, the scavenger of radicals free, obviously decreased amounts of ·OH radicals free in the culture media(Fig. 4).

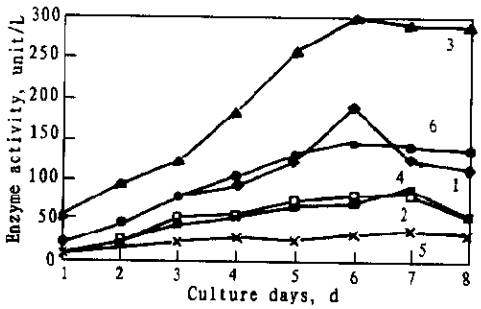

Fig. 3 Effect of veratryl alcohol and ascorbic acid on LiP and MnP activity

1. Lip activity without veratrayl alcohol; 2. Lip activity with as corbic acid; 3. Lip activity with veratryl alcohol; 4. MnP activity without veratryl alcohol; 5. MnP activity with ascorbic acid; 6. MnP activity with veratryl alcohol

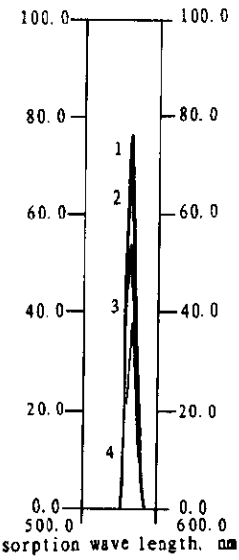

Fig. 4 Effect of veratryl alcohol and ascorbic acid on amounts of ·OH radical free

1. with veratryl alcohol; 2. with veratryl alcohol + ascorbic acid; 3. control; 4. with ascorbic acid

3.3 Cometabolic degradation of veratryl alcohol and biphenyl under nitrogen nutrition-rich condition

3.3.1 Degradation of veratryl alcohol

Degradation of veratryl alcohol increased to 90.7% in 8 days of culture under nitrogen nutrition-rich condition. Results from GC-MS analysis showed that the main intermediate product accumulated in the media was 2-methyoxy-phenol (retention time 13.18 min), this result was not the same to Marquez (Marquez, 1988), they reported that during the degradation of veratryl alcohol, 3, 4-dimethoxy radical were not changed, in the product of aromatic ring fission there were carboxyl radical presented. Result in our experiment showed that during the degradation process of veratryl alcohol a methyl radical might be removed first; when ascorbic acid, the scavenger of radicals free, existed the intermediate products accumulated were the 3,4-dimethoxy-benzenmethanol(retention 26.43 min) and 3,4-dimethoxyl-phenylacetone (retention time 27.76 min), these results suggested that degradation processes were different in the different reaction system under existence of veratryl alcohol or ascorbic acid separately(Fig.5).

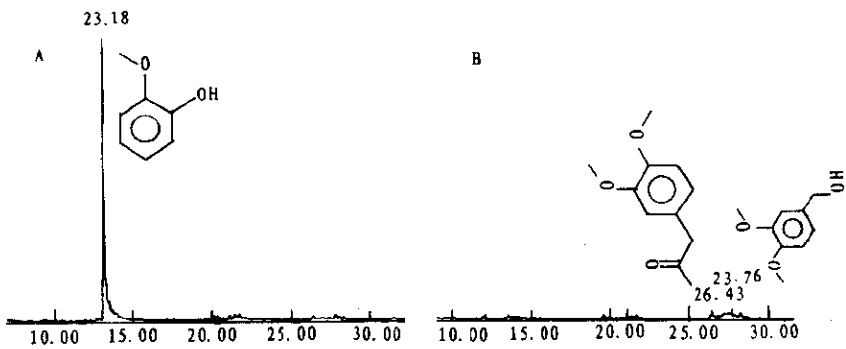

Fig.5 Degradation of veratryl alcohol (A) and effect of ascorbic acid (B) on it under the nitrogen nutrition-rich condition

3.3.2 Cometabolic degradation of veratryl alcohol and biphenyl under nitrogen nutrition-rich condition

When no veratryl alcohol was exerted, degradation velocity of biphenyl was very slow, the removal percentage was 37.5% during the culture period of 8 days, the main intermediate product accumulated was bis (2-ethylhexyl)-phthalate; veratryl alcohol promoted the degradation of biphenyl, the removal percentage reached 97.5% during the same period, and degradation intermediate product of veratryl alcohol itself detected at retention time of 13.18 min was not found. These results suggested that with the degradation of veratryl alcohol the rate of degradation of biphenyl was promoted, this was the phenomenon of cometabolism which was different from previous reports for degradation process for aromatic pollutants by white rot fungus. When the ascorbic acid was exerted, the degradation velocity decreased, the degradation ratio was only 9.7% during the culture period of 8 days(data not shown). The ascorbic acid is a scavenger of radical free, therefore, this results implicated that ·OH radical free played an important role in the

cometabolic degradation of veratryl alcohol and biphenyl(Fig. 6)

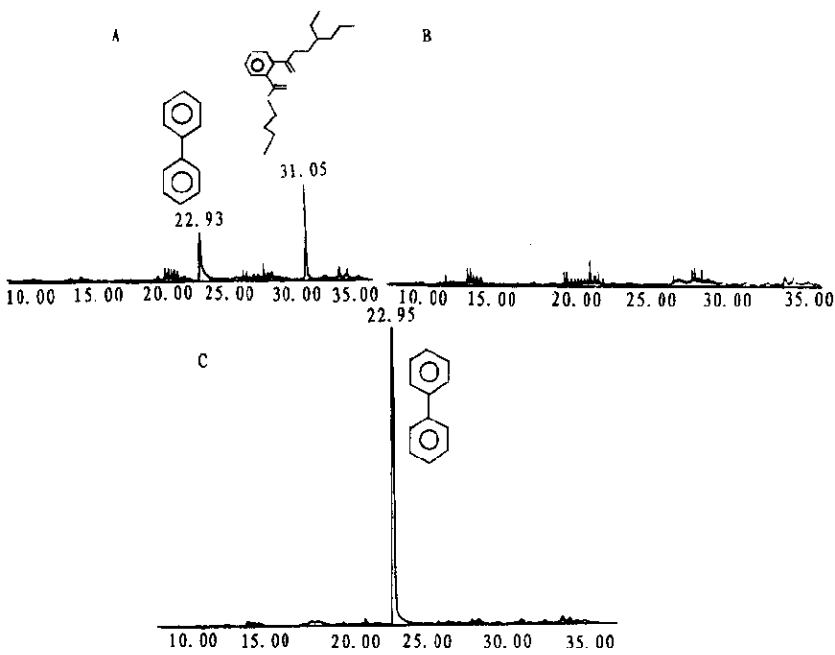

Fig. 6 Cometabolic degradation of veratryl alcohol and biphenyl under nitrogen nutrition-rich condition
a. Control; b. with veratryl alcohol; c. with ascorbic acid

4 Discussion

Tien and Kirk (Tien, 1983) reported that during the degradation process for lignin, production of LiP and MnP was promoted under carbon or nitrogen nutrition-limited condition, this results was considered to be the adaptability of white rot fungus to low concentration of nitrogen nutrition in the xylem tissue of dead plant, our results showed that the activity of LiP and MnP was related to the C/N value in the culture media, under the condition of nitrogen nutrition-rich condition, total activity of LiP increased with the increase of tartrate ammonium concentration when the glucose concentration was fixed to a certain degree in the culture media, but MnP was produced only under the condition of low N or C nutrition, this results implicated that mechanism of expression of LiP and MnP gene was different. In the environmental site of the soil or waters polluted by industrial effluent or agricultural herbicides which contains the recalcitrant aromatic pollutant, the rich nutrition of nitrogen often happens, under this condition veratryl alcohol can induce the production of LiP and MnP by white rot fungus, this may be useful to the application of white rot fungus into in situ bioremediation of environment polluted by recalcitrant aromatic compounds.

The result that ascorbic acid scavenger of radical free reduced the amount of ·OH radical free and inhibited the degradation of biphenyl by white rot fungus suggested that ·OH radial free in the

culture media of white rot fungus which was produced from the reaction of LiP and MnP with H_2O_2 played an important role in the degradation process of recalcitrant aromatic pollutants. Veratryl alcohol increased the amount ·OH radical free, this result reminded us of more effects that veratryl alcohol can exert in relating to the stability and protection of LiP and MnP from hurting by extra amount of H_2O than only inducing the production of LiP and MnP, thus it was suggested that addition of veratryl alcohol into the polluted site of environment may promote the degradation of recalcitrant aromatic pollutants in the process of in situ bioremediation, this hypotheses was already partly proven by the result of promotion to degradation process for recalcitrant aromatic pollutants biphenyl by veratryl alcohol and inhibition to this process by ascorbic acid in this experiment.

Veratryl alcohol can be degraded in the nitrogen nutrition-rich culture media. In the culture period in this experiment, the only intermediate product accumulated in the media was 2-methoxy phenyl, but under the cometabolic culture media of veratryl and biphenyl, the degradation speed of veratryl and biphenyl was more rapid, veratryl alcohol promoted the degradation of biphenyl with the degradation of itself, and intermediate product 2-methoxy phenol accumulated was not observed in the media, this cometabolic phenomenon was not reported ever before about the degradation of recalcitrant aromatic pollutants by white rot fungus besides other microbes. The veratryl alcohol induced the production as well as kept the stability of LiPs and MnPs, and it also promoted the degradation of other recalcitrant aromatic pollutants with the degradation of itself, but the mechanism still exposes to be studied further.

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