CHAPTER I

INTRODUCTION

1.1 Problem identification

Aromatic pollutants such as pentachlorophenol (PCP) and phenanthrene are ubiquitous environmental contaminants found in air, soil, and aquatic environments. Most of them are toxic to living organisms, and some of them and their metabolites are mutagenic and carcinogenic to humans. Although recently the production and utilization of some pollutants have decreased and even banned in some countries, it still causes an environmental problem at many locations due to their persistence in the environment (Choi and Aomine, 1974; Delaune et al., 1983; Butte et al., 1985; Kitunen et al., 1987). Therefore, the knowledge of mechanisms involving the bioremediation of this aromatic pollutant is critical.

Bioremediation by taking advantage of microorganisms and their enzymes is widespread nowadays. Among microorganisms, white rot basidiomycetous fungi are of the most interesting. The fungi offer a number of advantages for use in bioremediation. The key lignin degrading enzymes are extracellular, obviating the need to internalize the substrates and allowing the substrates of low solubility to be oxidized. Furthermore, the extracellular enzyme system of the white rot fungi enables these organisms to tolerate a relatively higher concentration of toxic pollutants than would otherwise be possible. The enzyme catalyzes the initial stages of attack on polyaromatic lignin (Reddy and Mathew, 2001). The extracellular reactions that they catalyze include lignin depolymerization as well as demethoxylation, decarboxylation, hydroxylation, and aromatic ring opening, many of the reactions result in oxygen activation, creating oxygen radicals that perpetuate the oxidative attack (Kirk and

Farrell, 1987; Schoemaker, 1990). Therefore, the non-specific enzyme properties make it more advantageous for white rot fungal enzyme to be capable of degrading a wide variety of pollutants. An example of lignin degrading enzyme is laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) produced as an extracellular enzyme by various white rot fungi. It belongs to a group of polyphenol oxidases. It catalyzes the four electron reduction of dioxygen to water by substrate molecules of phenolic origin without the generation of H_2O_2 (Mougin et al., 2003). Besides phenolic compounds, they can also attack non-phenolic aromatics with high redox potentials in the presence of small aromatic compounds such as 2,2'-azinobis-3-ethyl-benz-thiazoline-6-sulfonic acid (Hatakka, 1994; Thurston, 1994). Nevertheless, in the actual remediation, the degrading activities of these extracellular enzymes in the environment may be altered due to the interactions with humic substances (HS).

HS including humic acid (HA) and fulvic acid (FA) are up to 70% of soil organic carbon and up to 90% of dissolved organic carbon (Klavins and Serzane, 2000). Even though HS structure is exceeding complex, the major functional groups in HS are reviewed as oxygen containing and include carboxyls, alcoholic and phenolic hydroxyls, carbonyls, and methoxyls (Essington, 2004). Due to the similar functional groups of HS and oxidoreductase substrate, HS can compete for the oxidation and thus competitively inhibit the transformation of other compounds (Itoh et al., 2000). For example, Zavarzina et al. (2004) estimated inhibition constants for humic acids towards *Panus tigrinus* laccase. The K_i ranged from 0.003 μg/mL for HA from peat soils to 0.025 μg/mL for HA from chernozems. Nevertheless, HS could enhance the degradation rate of aromatic pollutants since it could interact with the pollutant and allowed them to be incorporated into fungal biomass (Nanny et al.,

1996). Moreover, HS can also both stimulate and inhibit enzyme activity depending on their origin sources as reported by Clause and Filip (1990, 1988).

Another possible role of HS is sorption of chemical including PCP and phenanthrene. It has been stated in connection with the structural characterization of HS, that PCP can be trapped and closely intercalated in the void of HS either without or with intermolecular hydrogen bonding between the hydrogen of the protonated carboxylic group (COOH) of HS and the phenolic oxygen of PCP (Schulten, 1996). Schwarzenbach, et al. (1993) reported that a main fate of nonvolatile, nonionic organic contaminants such as phenanthrene is sorption to soil or sediment organic matter.

How dissolved HS play a role in the system was the key question in the whole research. The possibilities of HS' role are 1) HS can deactivate enzymes 2) HS can compete with aromatic pollutants for enzyme 3) HS can protect aromatic contaminant. In this study, aromatic pollutants, pentachlorophenol (PCP) and phenanthrene were used as substrates. Purified laccase and crude fungal ligninolytic enzymes were used. HA, FA and DOM were employed in the research. This research is unique and different from others that have been previously reported because it clarifies the mechanisms how dissolved HS modifies pollutant degradation rates based on those three hypotheses. Even though a number of studies have explained the reaction of HS + pollutants by sorption studies (Paolis and Kukkunen, 1997; Peuvavouri et al., 2001; Salloum et al., 2002, Vacca et al., 2005), HS + enzymes (Ladd and Butler, 1975; Anh et al., 2002), pollutants + enzymes (Mileski et al., 1988; Lamar and Dietrich, 1990; Roy-Arcand and Archibald, 1991; Lamar et al., 1993; Reddy, 1995; Ullah et al, 2000), and HS + pollutants + enzymes (Zavarzina et al, 2004), none of the studies ever elucidated the mechanism of how HS affect the degradation rate of pollutants by

enzymes using the Michaelis-Menten equation for enzyme kinetics model and sorption isotherms. We expected that this information would enhance the potential for designing and improving aromatic pollutants bioremediation strategies. For example, HS could be an important variation by acting as an obstacle for bioremediation successfulness.

1.2 The purpose of the study

To elucidate the mechanisms by which HS interact with oxidoreductive enzymes and modify their degradation behavior of aromatic compounds.

1.2.1 Sub-objectives

1.2.1.1 To investigate the enzymatic degradation rate of PCP and phenanthrene described by K_m and V_{max} in the presence of dissolved HA and FA along with oxidoreductase enzymes.

1.2.1.2 To investigate changes of enzyme activity due to HS.

1.2.1.3 To study sorption efficiency of PCP and phenanthrene to HS.

1.2.1.4 To examine degradation mechanism of HS on PCP by purified laccase.

1.2.1.5 To examine degradation mechanism of HA, FA, and phenanthrene with crude fungal ligninolytic enzymes.

1.2.1.6 To examine degradation mechanism of phenanthrene and crude fungal ligninolytic enzymes modified by dissolved organic matter.

1.3 Hypotheses

1.3.1 HS disable (denatures/deactivates) the enzyme

HS and enzymes interact directly, forming enzyme-humic substance complexes. Moreover, HS may modify the enzymatic active center by changing the

quaternary or tertiary structure of the enzyme protein. Therefore, HS may disable enzyme activities (Muller-Wegener, 1988). As a result, no change in HS structure would be observed. To test whether HS disables enzyme, catechol, which is a positive substrate for laccase, would be added. If HS disables enzyme, catechol would not be enzymatically degradable.

1.3.2 HS is a competing enzymatic substrate

HS is a competing enzymatic substrate because HS contains numerous functional groups that are known to be oxidized by oxidoreductive enzymes. Therefore, some HS may act as analogous substrates and disturb the equilibrium of the enzymatic reaction (Muller-Wegener, 1988). If this hypothesis was true, modification of humic and fulvic molecules would be observed. UV-vis spectrophotometer could be used in the analysis of HA and FA's structure changes. Moreover, V_{max} in the presence and absence of HA and FA would be the same, while K_m was increased.

1.3.3 HS is not a competitive substrate but protects aromatic pollutants (sequestration)

Pollutants are strongly associated with HS by adsorption and oxidative coupling (solution-phase interactions). Aromatic pollutants are agglomerated into HS. After incorporation, humic molecules may not react with enzymes; therefore, HS help protect aromatic pollutants (Berry and Bord, 1985). Enzyme activities would be active and HS structure may or may not change while aromatic pollutants decrease. Moreover, we would observe higher K_m and lower V_{max} due to substrate was incorporated into HS.

1.4 Scope of Studies

1.4.1 In phase 1, purified laccase, pentachlorophenol, Aldrich humic acid (AHA), Leonardite humic acid (LHA), Suwannee River fulvic acid (SRFA), and Waskish peat fulvic acid (WFA) were incubated. PCP concentration were analyzed by GC-FID. Simple analytical instrument, UV-vis spectroscopy was used to investigate the change in specific absorption properties on HA and FA and to observe enzyme activity. Enzyme kinetics for contaminant and HS were studied. Moreover, equilibrium dialysis method would be employed to study of sorption capability of HS and PCP.

1.4.2 In phase 2, to examine role of AHA, LHA, SRFA, WFA and dissolved organic matter from paddy field soil with crude fungal oxidoreductive enzymes from *Agrocybe* sp. CU 43, the similar experiment was carried out except the enzyme from microorganisms was used instead of commercial enzymes and phenanthrene were used as a model aromatic pollutant.