CHAPTER II

LITERATURE REVIEW

2.1 Humic substances

Humic substances (HS) are yellow to black in color and have high molecular weight. As quoted by MacCarthy (2001) "HS comprise an extraordinarily complex, amorphous mixture of highly heterogeneous, chemically reactive yet refractory molecules that serve a key role in the Earth's ecological system, produced during early diagenesis in the decay of biomatter, and formed ubiquitously in the environment via processes involving chemical reaction of species randomly chosen from a pool of diverse molecules and through random chemical alteration of precursor molecules". Shinozuka et al. (2004) have reported number average molecular weight of peat and soil base humic and fulvic acids ranging from 2,900 to 10,500. Humic substances can be divided in three fractions. Humin is the fraction of HS that is not soluble in water at any pH. Humic acid (HA) is the fraction of HS that is not soluble in water under acidic conditions (below pH 2), but becomes soluble at greater pH. Fulvic acid (FA) is the fraction of HS that is soluble under all pH conditions (Aiken et al., 1985).

The structure of HS is far from determined. However, it has been revealed that the major building blocks of HS include biologically resistant molecular fragments that form most of the common precursor molecules of HS. The major structural units may be classified as residues originating from terrestrial plants (lignins), metabolites of algae (high molecular weight carbohydrates), and other structures. The specific major structural units of HS include benzenecarboxylic acids, phenolcarboxylic acids

as the major units of the humus core bridged with hydrogen-carbon links, and structures of carbohydrate transformation. Contemporary concept of HS is summarized in Figure 2.1. The structure of HS substantially depends also on the humification conditions, that is, on the process in the corresponding environment (Klavin and Serzane, 2000).

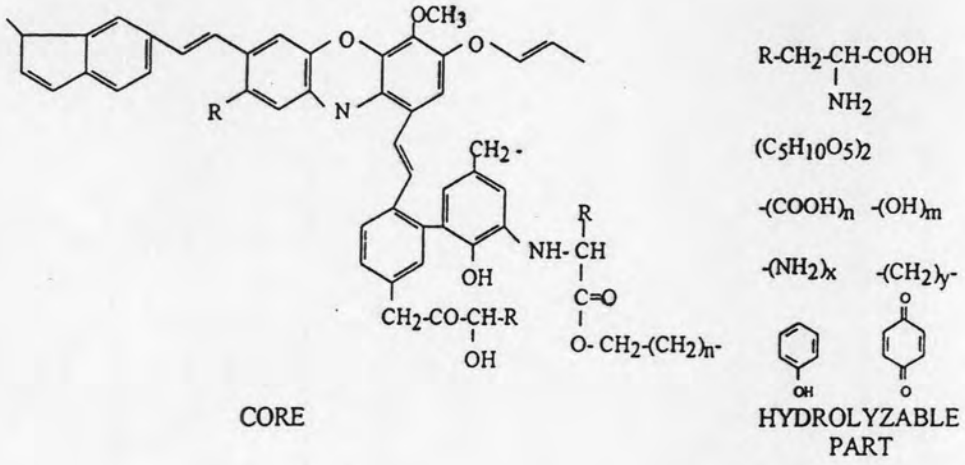


Figure 2.1 Structure model of an elemental building block of soil humic acid (Klavin and Serzane, 2000)

Four types of HS including Aldrich humic acid (AHA), Leonardite humic acid (LHA), Suwannee River fulvic acid (SRFA), and Waskish peat fulvic acid (WFA) were used in the experiment. The reasons for using those HS as model are (1) they are available worldwide (2) all materials are carefully prepared and homogenized and (3) they are well characterized. These HS are also difference in % aromaticity and molecular weight. Their characteristics are shown in Table 2.1.

Table 2.1 Chemical and physical properties of particular humic substances

Humic substances	Sources	% Aromaticity	% Carboxyl	% C	% O	% N	MW (Dalton)
Aldrich humic acid (AHA)	Lignite, Aldrich company	41.0[a]	19[a]	65.3[d]	25.1[d]	-	4,731[e]
Leonardite humic acid (LHA)	Gascoyne Mine, North Dakota, USA	58.0[b]	7.5[b]	63.8[b]	31.3[b]	0.8[b]	18,700[f]
Suwannee River fulvic acid (SRFA)	Suwannee River, South Georgia, USA	24.0[c]	12.2[b]	53.0[b]	43.9[b]	0.5[b]	2,519[e]
Waskish peat fulvic acid (WFA)	Pine Island Bog, Minnesota, USA	36.0[b]	-	53.6[b]	38.5[b]	0.3[b]	11,950[g]

a: Ashley, 1996; b: International Humic Substances Society (IHSS); c: Thorn et al., 1989; d: Malcolm and MacCarthy, 1986; e: O'Loughlin et al., 2000; f: Beckett et al., 1987; g: Perminova et al., 2003.

2.2 Aromatic pollutants

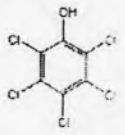
Environmental pollutants are a serious concern worldwide because of hazards they pose to the health of humans and animals. An estimated 80 billion pounds of hazardous organopollutants are produced annually by the chemical, agricultural, oil, paper, texile, aerospace, and other industries in the USA alone (Aust, 1990). Only 10% of these wastes are believed to be disposed of in an environmentally safe manner (EPA, 1988; Fernando & Aust, 1994). Aromatic pollutants are one of the major groups of toxic chemicals responsible for environmental contamination and an important potential risk to human health. Two pollutants of interest in this study were pentachlorophenol (PCP) and phenanthrene. Their physical properties are shown in Table 2.2.

Pentachlorophenol is a chlorinated hydrocarbon insecticide and fungicide. The widespread use of PCP in agriculture and industry as fungicides, insecticides, herbicides and disinfectants, together with their acute toxicity, has led to the contamination of both terrestrial and aquatic ecosystems. As a consequence, PCP is now banned in several countries, including Thailand (Thailand Pesticides Database, 2006). PCP is a very toxic compound, and is labeled with a DANGER signal word. Its LD_{50} is 25 – 200 mg/kg (oral, rats), 130 mg/kg (oral, mice and rabbits), 105 mg/kg (dermal, rabbits), 96 – 320 mg/kg (dermal, rats), 261 mg/kg (dermal, mice), 10 – 225 mg/kg (inhale, rats), 10 – 225 mg/kg (inhale, mice). The exact dose required to produce illness in humans is not known. Skin penetration is the most dangerous route of exposure, but inhalation or ingestion of PCP may also cause toxicity. Acute exposure of PCP can cause elevated temperature, profuse sweating, dehydration, loss of appetite, decreased body weight, nausea, uncoordinated movement and coma. For chronic toxicity, animal experiments suggest that chronic exposure to pure PCP may affect reproduction, induce birth defects, and cause acne and other skin diseases. When PCP enters soil, it is degraded by sunlight and bacteria, and can leave the upper soil layer by evaporation and leaching into groundwater (Extoxnet, 2008). Once reaching water, PCP may be degraded by sunlight or microorganisms or bind to sediments and suspended particles in water. Dissolved in water, it does not evaporate to a significant degree (Extoxnet, 2008).

Phenanthrene is a polycyclic aromatic hydrocarbon (PAH) that can be derived from coal tar. Currently, there is no commercial production or use of this compound. Phenanthrene is ubiquitous in the environment as a product of incomplete combustion of fossil fuels and wood and has been identified in ambient air, surface and drinking water, and in foods. Phenanthrene is absorbed by oral and dermal exposure. Although

a large amount of literature exists on the toxicity and carcinogenicity of PAHs, primarily benzo[a]pyrene, toxicity data for phenanthrene are very limited. No human data were available that addressed the toxicity of phenanthrene (RAIS, 2008). Based on no human data and inadequate data from animal bioassays, US-EPA has placed phenanthrene in group D, not classifiable as to human carcinogenicity (RAIS, 2008).

Table 2.2 Physical properties of the two selected compounds

Properties	Pentachlorophenol	Phenanthrene
Molecular Formula	C_6HCl_5O	$C_{14}H_{10}$
Molecular Structure		
Log K_{oc}	4.5	4.57
Molecular weight (g/mol)	266.35	178.23
Water Solubility (mg/L @ 20°C)	14	1.18 – 1.65
Log K_{ow}	5.01	4.36
Vapor Pressure(mmHg at 25°C)	1.1×10^{-4}	$1.4 \times 10^{-3} - 7.9 \times 10^{-4}$
pK_a	4.7	N/A

N/A = Not available

Source: http://www.atsdr.cdc.gov, 2006, www.nature.nps.gov, 2006

2.3 Bioremediation potential of white rot fungi

Most fungi are robust organisms and are generally more tolerant to high concentrations of polluting chemicals than are bacteria, which explains why fungi have been investigated extensively since the mid-1980s for their bioremediation capabilities. Environmental pollutants are more concerned and traditional methods of disposing of hazardous wastes (physical, chemical, and thermal treatments and land

filling) have not always been efficacious. It has been estimated that it costs about one trillion dollars to decontaminate toxic waste sites in the USA alone using traditional waste disposal methods (Barr & Aust, 1994). Considering these staggering costs for cleaning up the environment, an alternative, rapid, efficacious and cost-effective method is needed. One method that has become increasingly popular for decontamination of the environment has been bioremediation. The use of indigenous or suitable introduced microorganisms at contamination sites often provides an efficient and economically attractive solution to the pollution problem. One of the early reports indicated that lignin-degrading white rot fungi, as exemplified by *Phanerochaete chrysosporium*, can degrade an extremely diverse group of environmental pollutants (Bumpus et al., 1985). Since then, there has been intense worldwide research to unravel the potential of white rot fungi in bioremediation. The ability of white rot fungi to degrade a wide spectrum of environmental pollutants sets them apart from many other microbes used in bioremediation.

White rot fungi are wood-degrading basidiomycetes and are among the most active degraders of lignin, the key structural polymer of woody plants. Lignin is a highly complex, three-dimensional, amorphous, heteropolymer and consists of phenylpropanoid monomer units that are randomly linked to each other in a variety of C-C and C-O linkages. Furthermore, the chiral carbons in lignin occur in both L and D configurations. Therefore, lignin is one of the most difficult biopolymers to be degraded by microbial enzymes. The complexity of the lignin polymer and its sterochemical irregularity results from the free radical mechanism of lignin synthesis seen in woody plants (Kirk and Farrell, 1987; Boominathan and Reddy, 1992; Barr and Aust, 1994). It has been hypothesized that lignin degradation must also involve a non-specific and non-stereoselective mechanism. Extensive research since the early

1980s has shown that the white rot fungi have developed unique non-specific enzyme systems with the ability to attack not only lignin but also a broad spectrum of halogenated and non-halogenated aromatic compounds as well as some non-aromatic organopollutants.

White rot fungi offer a number of advantages for use in bioremediation (Reddy and Mathew, 2001). The key enzymes of the lignin degradation system are extracellular, obviating the need to internalize the substrates and allowing substrates of low solubility to be oxidized. Furthermore, the extracellular enzyme system of the white rot enables these organisms to tolerate a relatively high concentration of toxic pollutants than would otherwise be possible. White rot fungi catalyze degradation of lignin as well as pollutants using a non-specific free radical mechanism and are, consequently, capable of degrading a wide variety of pollutants. The constitutive nature of the key enzymes involved in the lignin degrading system obviates the need for these organisms to be adapted to the chemical being degraded. White rot fungi are also ubiquitous in nature. Although they degrade lignin, they cannot utilize it as a source of energy for growth and instead require cosubstrates such as cellulose or other carbon sources. The preferred substrates for growth of white rot fungi in nature are lignocellulosic substrates. The examples of lignocellulose normally added to contaminated sites to enhance fungal growth are corn cobs, straw, peanut shells, and sawdust (Reddy and Mathew, 2001).

2.4 Ligninolytic enzymes

The lignin-degrading enzymes of white rot fungi consist of a battery of enzymes that catalyze oxidation of xenobiotics in addition to their ability to degrade

lignin. The lignin-degrading enzymes cleave the carbon-carbon and carbon-oxygen bonds of the lignin molecule regardless of the chiral conformations of the lignin molecule (Fernando and Aust, 1994). This manner of bond fission may result partially from the free radical mechanism of lignin degradation employed by white rot fungi (Kirk and Farrel, 1987; Aust, 1990; Boominathan and Reddy, 1992; Fernando and Aust, 1994). In addition, free radical species generated during the degradation process (of either lignin or organopollutants) may serve as secondary oxidants, which may, in turn, mediate the oxidation of other compounds away from the active sites of the enzymes (Barr and Aust, 1994). Nitrogen deficiency was observed to initiate the degradation of pollutants by *P. chrysosporium* (Bumpus et al., 1985; Barr and Aust, 1994; Reddy, 1995).

Lignin degrading system in *P. chrysosporium* is expressed during secondary metabolism in response to starvation for nutrients such as nitrogen and carbon. Ligninolytic peroxidases, which are believed to be involved in lignin degradation by this organism, are completely suppressed in media containing high levels of nitrogen or carbon. The three major families of lignin-modifying enzymes that are believed to be involved in lignin degradation are laccases, lignin peroxidases, and manganese peroxidases (Reddy and Mathew, 2001). Some white rot fungi produce all three classes of lignin modifying enzymes while others produce different combinations of the three (Cullen and Kersten, 1992; Reddy, 1993; Reddy and D'Souza, 1994; Thurston, 1994).

2.4.1 Laccase

An example of ligninolytic enzyme is laccase, EC 1.10.3.2, *p*-diphenol:dioxygen oxidoreductase, are part of a group of enzymes termed the multicopper enzymes. Laccase contains four copper atoms, which are distributed into three sites (T1, T2, T3, Figure 2.2) according to their spectroscopic properties. The T1 site contains the type 1 blue copper (Cu1), which coordinates a cysteine, and is responsible for the blue color of the enzyme. The T2 site contains a type 2 copper (Cu2). In the T3 site, Cu3a and Cu3b are strongly coupled. Laccase usually comprise 520 – 550 amino acids, with a molecular weight ranging from 60 – 80 kDa. It is produced in the cells, and is secreted and mainly accumulates in the outside of the hyphal filaments (Mougin et al., 2003). Kinetics and mechanisms of action of copper oxidases has been published by Messerschmidt (1997). In a first step, a reducing organic compoound binds to the active site and it is transformed into radical forms. Then, the mononuclear T1 site extracts electrons from the reducing substrate and mediates their transfer to the trinuclear T2/T3 site where molecular dioxygen is reduced to water (Figure 2.2). Finally, the radical compounds formed from the substrate are released. They are very reactive and able to interact with themselves or with other compounds present in the vicinity, thus inducing numerous reactions.

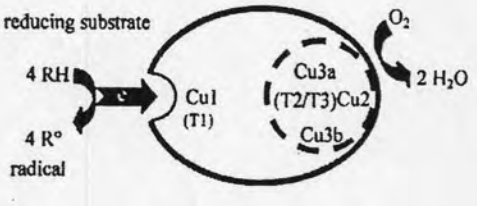


Figure 2.2 Schematic mechanism of action of fungal laccases (Mougin et al., 2003)

2.4.2 Peroxidases

Lignin peroxidase (EC 1.11.1.7) is extracellular, glycosylated heme proteins that catalyze H_2O_2-dependent one-electron oxidation of lignin-related aromatic compounds to aryl cation radicals, leading to a variety of end products through non-enzymic reactions. Lignin peroxidase has a higher redox potential (IP $\leq$ 7.35) than do most peroxidases and appear to oxidize a greater range of chemicals than many other peroxidases. Manganese peroxidase (EC 1.11.1.7) is extracellular glycosylated heme proteins that catalyze H_2O_2-dependent oxidation of Mn^{2+} to Mn^{3+}. It is the Mn^{3+} state of the enzyme that actually mediates the oxidation of phenolic substrates, while non-phenolic compounds are oxidized via cation radicals (Kirk and Farrell, 1987; Hammel, 1992; Barr and Aust, 1994; Reddy and D'Souza, 1994).

It is important to know that the key step in lignin degradation by laccase or peroxidases involves the formation of free radical intermediates, which are formed when one election is removed or added to the ground state of a chemical. Such free radicals are highly reactive and rapidly give up or abstract an electron from another chemical. This free radical mechanism provides the basis for the non-specific nature of degradation of a variety of structurally diverse pollutants (Barr and Aust, 1994).

2.5 Biodegradation of chlorophenol and PAHs using ligninolytic enzymes

Much attention has been directed towards the use of microorganisms and their enzymes for bioremediation of the pollutants including aromatic pollutants. With increasing number of studies, it is clearly that fungi, and in particular white rot fungi, are able to degrade a large number of pollutants. White rot fungi are characterized by their ability to degrade lignin in wood, which is a structurally complex (Kirk et al.,

1992). The unique abilities of ligninolytic enzymes such as laccase, lignin peroxidase secreted by those white rot fungi are highly potent, non-specific, non-stereoselective, extracellular enzymes. Since lignin contains numerous substructures that are also found in common aromatic pollutants, example phenolics and biphenyls, the fungi have a potential to degrade both lignin and aromatic pollutants (Kirk et al., 1992). A group of chlorophenol and polycyclic aromatic pollutants (PAHs) would be focus on the review.

2.5.1 Chlorophenols

Over decades, chlorophenols have been generated for application in agriculture and are important constituents of paper-mill effluents (Huynh et al., 1985; Aust, 1990). Large-scale use of PCP as a wood preservative and as a fungicide/herbicide has led to the contamination of terrestrial and aquatic ecosystems and it is one of the priority pollutants listed by the US Environmental Protection Agency (EPA, 1988). A number of studies have shown that PCP is rapidly degraded by *P. chrysosporium* under nitrogen-limiting secondary metabolic conditions (e.g. ligninolytic conditions) while degradation was inhibited in high-nitrogen media (e.g. non-ligninolytic conditions), suggesting the involvement of lignin degrading enzymes in PCP degradation by *P. chrysosporium* (Mileski et al, 1988). PCP degradation of 20 – 50% was reported in nitrogen-limited static cultures (Reddy, 1995). Moreover, studies have reported the abilities of ligninolytic enzymes to PCP degradation. It was found that laccase from *Trametes versicolor* is capable of oxidizing PCP with a high K_m value (Ullah et al., 2000). K_m is a Michaelis-Menten constant. It shows the substrate-enzyme affinity. They stated that the reaction of laccase with PCP must be solely with the phenolic group since they found no detectable free chloride. This

result was confirmed by the work of Roy-Arcand and Archibald (1991). The primary product of PCP reaction with laccase was high molecular weight polymer, which appeared stable under aqueous conditions both in alkaline and acid. Also, trace quantities of highly toxic tetrachlorobenzoquinone were found. Several polyphenoloxidases, such as lignin, manganese, horseradish peroxidases, and laccase, excreted from white-rot fungi have the ability to catalyze phenolic compounds, including PCP, forming the polymeric compounds. From 88 – 90 % of PCP is depleted after 60 days, depending on the fungus (Lamar and Dietrich, 1990; Lamar et al., 1990, 1993). A small part of it is transformed to carbon monoxide and water (2 – 9 %) (Lamar et al., 1990). A study of PCP degradation by *P. chrysosporium*, Reddy and Gold (2000) reported that following initial removal of the 4-chlorine of PCP by peroxidative dechlorination and reduction of the resultant tetachlorobenzoquinone to form 2,3,5,6-tetrachloro-1,4-dihydroxybenzene, the remaining chlorine substituents of this intermediate were removed by successive reductive dechlorination steps (Figure 2.3).

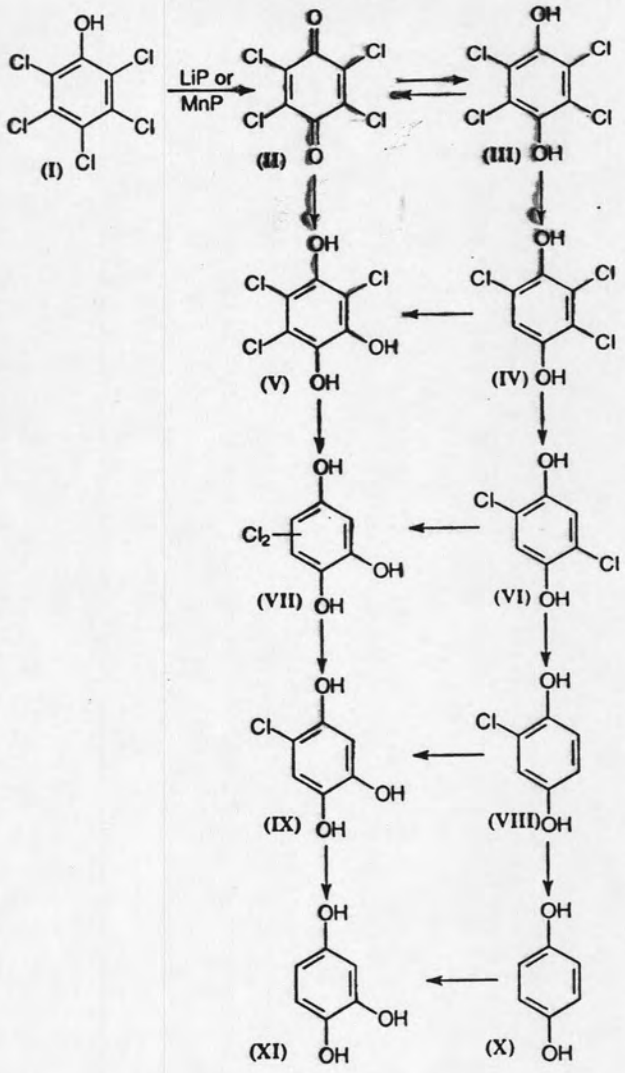


Figure 2.3 Proposed pathways for the degradation of PCP by *P. chrysosporium*. I, PCP; II, tetrachlorobenzoquinone; III, 2,3,5,6-tetrachloro-1,4-dihydroxybenzene; IV, chlorodihydroxybenzene; V, 3,5,6–trichlorotrihydroxybenzene; VI, 2,5-dichlorodihydroxybenzene; VII, dichlorotrihydroxybenzene; VIII, 2-chloro-1,4-dihydroxybenzene; IX, chlorotrihydroxybenzene; X, *p*-hydroquinone; XI, trihydroxybenzene; LiP, lignin peroxidase; MnP, manganese peroxidase. (Reproduced from Reddy and Gold, 2000)

2.5.2 Polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are widespread, hazardous environmental pollutants that are released to air, soil, water and marine environments by the burning of fossil fuels and wood, coal mining and oil drilling (Fernando and Aust, 1994). Several of them are carcinogenic and mutagenic (Zhang and Jenssen, 1994). Enzymes from white rot fungi have reported to have the capability to metabolize phenanthrene. Sutherland et al. (1991) studied the metabolism of phenanthrene by *P. chrysosporium* after raising them for 7 days at 37°C in a medium containing malt extract, D-maltose, yeast extract, and Tween 80. Based on UV-absorption, mass spectrometry data and ^{13}C nuclear magnetic resonance spectra, it was shown that the chemical was converted to phenanthrene trans-9,10-dihydrodiol, phenanthrene trans-3,4-dihydrodiol, 9-phenanthrol, 3-phenanthrol, 4-phenanthrol, and the novel conjugate 9-phenanthryl β-D-glucopyranoside. Nevertheless, since lignin peroxidase was not detected, they suggested that the involvement of monooxygenase and epoxide hydrolase activity in the initial oxidation of phenanthrene by *P. chrysosporium*. Hammel et al. (1992) also demonstrated that *P. chrysosporium* metabolized phenanthrene initially to phenanthrene 9,10-quinone and then to a ring cleavage product, 2,2'-diphenic acid, under ligninolytic conditions (Figure 2.4). Lipid peroxidation by the manganese peroxidase of *P. chrysosporium* has also been implicated in phenanthrene degradation (Moen and Hammel, 1994). Han et al. (2004) explained the mechanism of phenanthrene metabolism by incubating the chemical with 5 units of purified laccase of *Trametes versicolor*. They found that phenanthrene was not transformed. The addition of redox mediator; 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) or 1-hydroxybenzotriazole (HBT) to the reaction mixture resulted in an oxidation of phenanthrene by laccase about 40% and

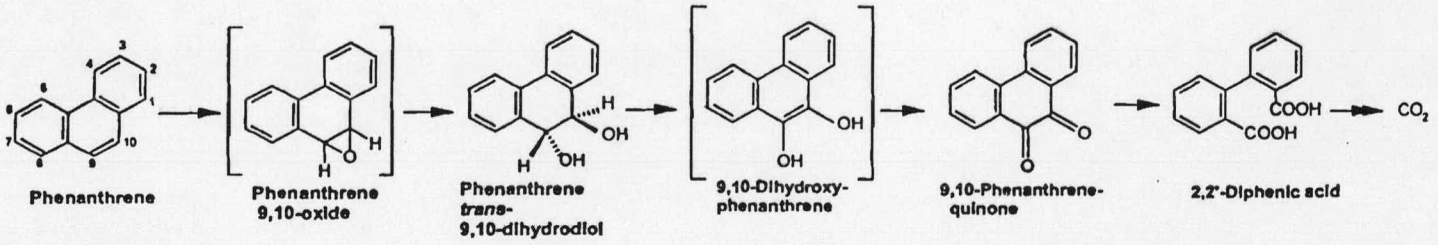


Figure 2.4 Metabolism of phenanthrene by ligninolytic enzymes (Hammel et al., 1992)

30%, respectively. They discussed that laccase is a polyphenol oxidase and is commonly limited to phenolic compounds with a very low ionization potential (IP). The IP values, referring to the energy required to remove an electron and to form a cation radical. Phenanthrene has an IP value of 8.19 eV. while laccase has 7.45 eV. Therefore, phenanthrene cannot be a substrate of laccase. The result is confirmed by Lau et al. (2003) who found that phenanthrene was the least degradable compound among PAHs used in the experiment by using spent mushroom compost to remediate PAH-contaminated samples. Disappearance of PAHs showed a strong correlation with their IP. The IPs (eV) of four PAHs were reported as naphthalene, 8.12; phenanthrene, 8.03; benzo[a]pyrene, 7.21; and benzo[*g, h, i*]perylene, 7.31. A one-electron oxidation of PAHs can take place by peroxidases (IP $\leq$ 7.35), laccase (IP $\leq$ 7.45), Mn-dependent peroxidase (IP $\leq$ 8.19), and ligninase (IP $\leq$ 7.55). Even though phenanthrene cannot be a substrate of those oxidoreductase enzymes, in the presence of appropriate low-molecular-mass compounds called mediators, laccase is able to oxidize various aromatic compounds (Han et al., 2004). In their study, the laccase of *Trametes versicolor* could transform phenanthrene efficiently with HBT (30% degraded), and addition of ABTS showed an even higher transformation rate of phenanthrene during 2 hour of reaction.

2.6 Effect of humic substances on enzymes and enzyme kinetics

2.6.1 Effect of HS on enzymes

The inhibition or stimulation of the activity of various enzymes by HS has been reviewed. For a series of proteolytic enzymes no uniform effect by HS could be determined (Ladd and Butler, 1975): the activities of carboxypeptidase A, trypsin, and pronase B were inhibited, while papain, subtilopeptidase A, termolysin, and ficin were stimulated. HS had no effect on tyrosin and phaseolin. HA and its monomeric constituents either increase (Wang et al., 1992) or inhibit oxidoreductases activity (Kang et al., 2002). Decrease in laccase activity in HA solution or organo-clay suspension by complex formation with HA or adsorption on to clay surfaces was reported by Clause and Filip (1990) and Ruggiero and Radogna (1984). Moreover, Holman et al. (2002) proposed that HA can cause inactivation of laccase enzyme.

The interactions of HS with enzymes can be summarized followed Műller-Wegener (1988) as shown in Figure 2.5:

1. HS and enzymes interact directly. Binding mechanisms extend from adsorptive or steric effects to atomic bonds (e.g., an acid amide or ester linkage). HS are not only able to modify the active center by changing the quaternary or tertiary structure of the enzyme protein but can also act directly on the active sites.

2. HS comprise a multitude of different structures and do not have a single molecular weight. Therefore, some HS may act as analogous substrates and disturb the equilibrium of the enzymatic reaction.

3. The cation exchange properties of HS result in fixation of bivalent cations often used as cofactors for enzymatic catalysis or for stabilization of the structure of the protein molecule.

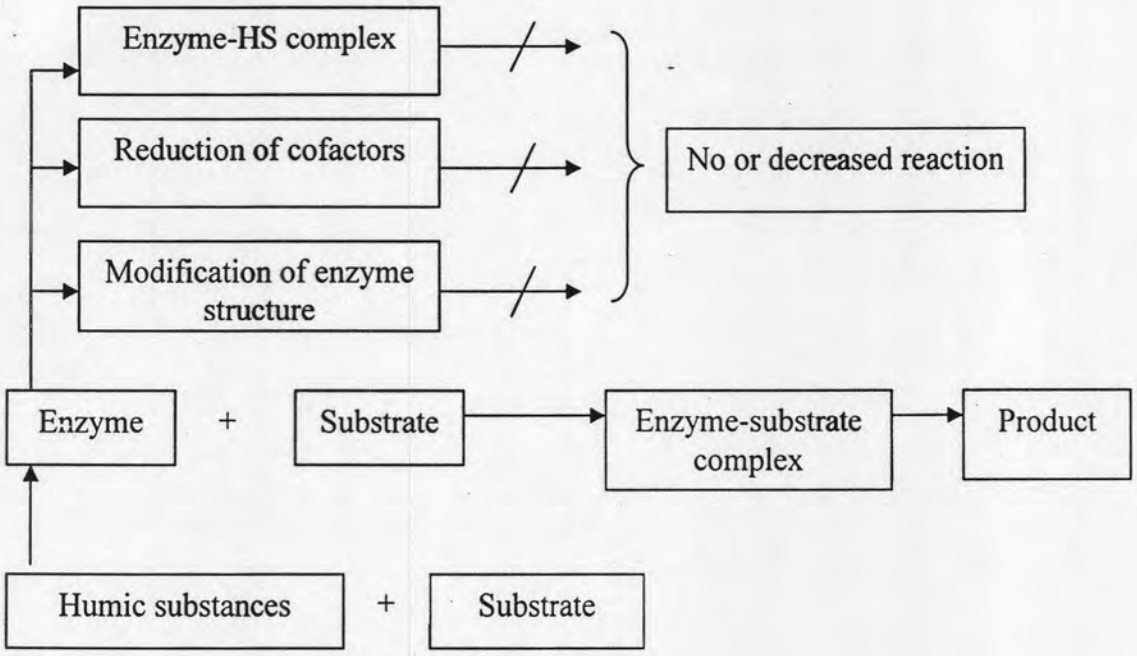


Figure 2.5 Possible interactions of humic substances and enzymes

2.6.2 Effect of HS on enzyme kinetics

Enzyme kinetics studies are one of the major research objectives in this study. Initial reaction rate of the model pollutants degraded by ligninolytic enzymes would be studied. Laccase from *Trametes versicolor* was previously studied by Ullah et al. (2000) and shown to have the ability to degrade PCP Chupungars et al. (2008) recently reported the ability of white rot fungal *Agrocybe* sp. CU 43 to transform a series of PAHs including phenanthrene. However, they did not explain kinetics by which the enzymes react with the pollutants. This research would be focus on enzyme

kinetics model using Lineweaver-Burke plot to compare degradation rate of PCP and phenanthrene in the presence and absence of HS.

The degradation rate of the pollutants might be modified due to the presence of HS in the system of interest. HS which contain numerous functional groups known to be substrates of ligninolytic enzymes were assumed to act as inhibition of pollutant enzymatic degradation. Due to the similar functional group of HS and enzyme 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. Although some researchers have studied on inhibitory effect of HS on enzymes, no one has reported inhibition of HS on enzyme degradation of PCP and phenanthrene. Moreover, the mechanism of HS on degradation rate of PCP and phenanthrene has never been elucidated.

2.6.2.1 Fundamental of enzyme kinetics

The kinetics of simple enzyme catalyzed reaction was characterized by the Michaelis-Menten equation (1)

$$v = V_{max}S / (K_m + S) \quad (1)$$

It shows the relationship between the velocity or rate of enzyme catalyzed reaction (v) and substrate concentration (S). K_m is the Michaelis-Menten constant and V_{max} is the maximum reaction rate (Figure 2.6). If v is equal to $1/2V_{max}$ then $S = K_m$. At low substrate concentrations, the rate of an enzymatic reaction is of first-order and is directly proportional to substrate concentration ($v = (V_{max}/K_m).S$) while at high

substrate concentration the rate becomes independent of substrate concentration, is represented by zero order kinetics ($v = V_{max}$) and almost all of the enzyme molecules are bound to substrate. Because V_{max} cannot be determined accurately by plotting v and S, the Lineweaver-Burke plot is introduced an analysis of enzyme kinetics by a straight line double reciprocal plot of 1/v against 1/S (Figure 2.7) with a slope of K_m/V_{max} and a y-intercept on the ordinate at $1/V_{max}$, from the relationship shown in equation (2)

$$1/v = 1/V_{max} + (K_m/V_{max})(1/S) \qquad (2)$$

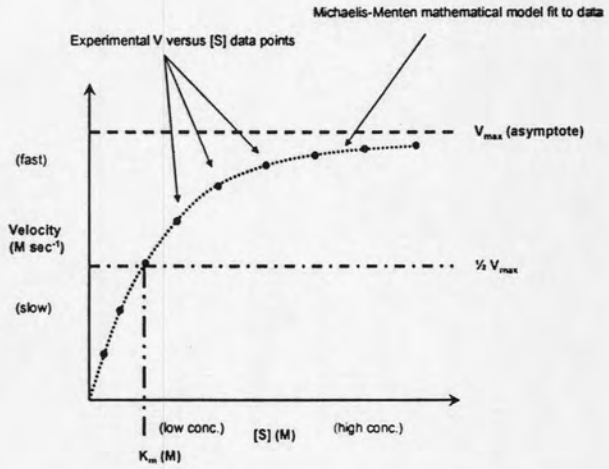


Figure 2.6 A typical Michaelis-Menten curve representing changes in velocity of an enzyme catalyzed reaction with respect to substrate concentration (Blaber, 2006)

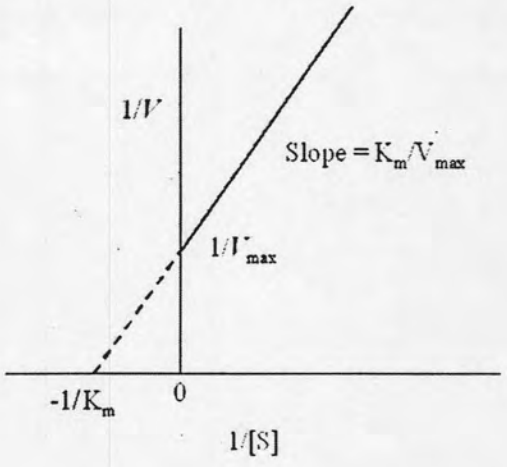


Figure 2.7 Lineweaver-Burke linear relationship (Blaber, 2006)

2.6.2.2 Fundamental of enzyme inhibition

An inhibitor is a compound which interacts with an enzyme to slow down its rate of reaction. Humic substance which contains a numerous functional groups could be a substrate for oxidoreductive enzyme and act as an inhibitor. Therefore, HS was able to slow down the enzymatic degradation rate of aromatic pollutants. The inhibition can be reversible or irreversible. Reversible enzyme inhibition can be competitive, uncompetitive, or linear mixed type, each affecting K_m and V_{max} in a specific fashion. This section would explain reversible inhibition of competitive, uncompetitive and linear mixed types. Moreover, the estimation of the enzyme-inhibitor dissociation constant (K_i) was discussed. A system occupied a stronger inhibitor or a lower K_i has a slower enzymatic degradation rate.

2.6.2.2.1 Competitive inhibition

A competitive inhibitor is a compound which bears a close structural and chemical similarity to the substrate of the enzyme. Due to this similarity, the inhibitor binds to the active site in place of the substrate.

$$\begin{array}{ccccc} & & K_m & & K_{cat} & \\ E + S & \leftrightarrow & ES & \rightarrow & E + P \\ + & & & & \\ I & & & & \\ \updownarrow & K_i & & & \\ EI & & & & \end{array}$$

The rate equation for the formation of product, the dissociation constants for enzyme-substrate (ES) and enzyme-inhibitor (EI) complexes, and the enzyme mass balance are, respectively:

$$v = K_{cat}[ES] \qquad (3)$$

$$K_m = [E][S]/[ES] \qquad K_i = [E][I]/[EI]$$

$$[E_T] = [E] + [ES] + [EI] = [E] + [E][S]/K_m + [E][I]/K_i$$

Normalization of the rate equation (3) by total enzyme concentration ($v/[E_T]$) and rearrangement result in the following expression for the velocity of an enzymatic reaction in the presence of a competitive inhibitor, where $V_{max} = K_{cat}[E_T]$

$$v = (V_{max}[S]) / (K'_m + [S])$$
$$= (V_{max}[S]) / (\alpha K_m + [S]) \qquad (4)$$

Where K'_m corresponds to the apparent enzyme-substrate dissociation constant in the presence of an inhibitor. In the case of competitive inhibition, $K'_m = \alpha K_m$, where

$$\alpha = 1 + [I] / K_i \qquad (5)$$

However, because the substrate and inhibitor are not identical the enzyme is unable to convert the inhibitor into product. The competitive inhibitor affects K'_m. K_m is an indication of enzyme-substrate affinity. In the presence of a competitive inhibitor some enzyme molecules will exist as free enzymes, resulting in having normal affinity. Others act as enzyme-inhibitor complexes, which have zero affinity because they are totally incapable of binding substrate. K'_m is measuring the overall affinity of the enzyme in the reaction mixture which will be an average value between the normal (K_m) and zero affinity enzymes which will be less than the normal value. So a competitive inhibitor reduces enzyme-substrate affinity, or increase K'_m. However,

the competitive inhibitor does not affect V_{max}. V_{max} is the velocity at very high substrate concentration. Under this condition, the inhibitor is competed out by the substrate and does not inhibit the enzyme at all. So competitive inhibitors do not slow the reaction at high substrate concentration and there is no change in V_{max} (Figure 2.8 – 2.9).

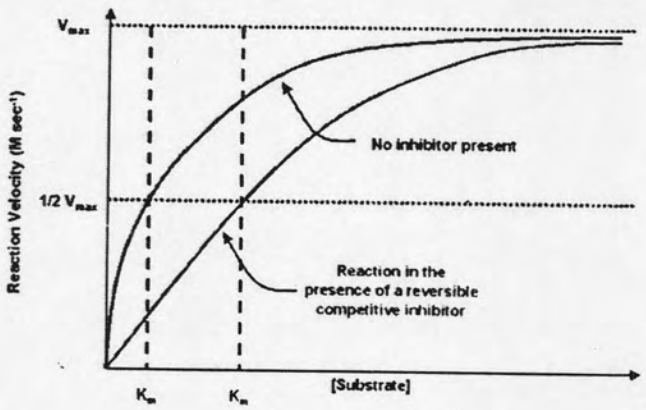


Figure 2.8 A Michaelis-Menten curve representing changes in velocity of an enzyme catalyzed reaction with respect to substrate concentration in the presence and absence of competitive inhibitor (Blaber, 2006)

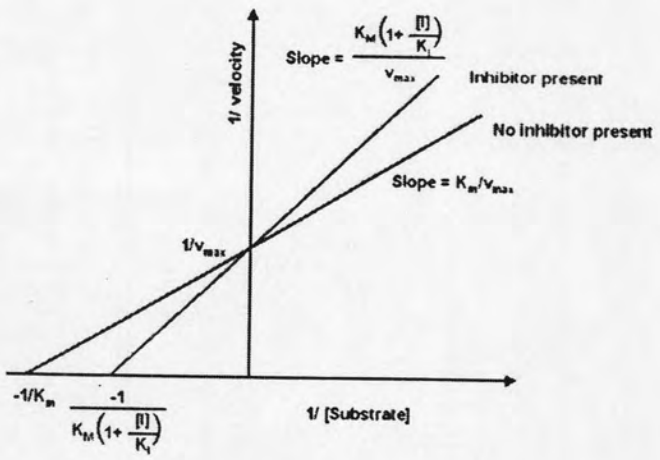


Figure 2.9 Lineweaver-Burke linear relationships with and without competitive inhibitor (Blaber, 2006)

2.6.2.2.2 Uncompetitive inhibition

In this type of reversible inhibition, a compound interacts with the enzyme-substrate complex at a site other than the active site,

$$E + S \overset{K_m}{\leftrightarrow} ES \overset{K_{cat}}{\rightarrow} E + P$$

$$ES + I \updownarrow K_i \; ESI$$

This results in an apparent decrease in both V_{max} and K_m. The apparent increase in affinity of enzyme for substrate is due to unproductive substrate binding, resulting in a decease in free enzyme concentration. Half-maximum velocity, or half-maximal saturation, will therefore be attained at a relatively lower substrate concentration. The rate equation for the formation of product, the dissociation constants for enzyme-substrate (ES) and ES-inhibitor (ESI) complexes and the enzyme mass balance are, respectively,

$$v = K_{cat}[ES] \qquad (6)$$

$$K_m = [E][S]/[ES] \qquad K_i = [ES][I]/[ESI]$$

$$[E_T] = [E] + [ES] + [ESI] = [E] + [E][S]/K_m + [E][S][I]/K_mK_i$$

Normalization of the rate equation (6) by total enzyme concentration ($v/[E_T]$) and rearrangement result in the following expression for the velocity of an enzymatic reaction in the presence of a competitive inhibitor, where $V_{max} = K_{cat}[E_T]$

$$v = (V'_{max}[S]) / (K'_m + [S])$$

$$= \quad (V_{max}/\alpha)[S] / (K_m/\alpha + [S]) \qquad (7)$$

Where V'_{max} and K'_m correspond respectively to the apparent enzyme maximum velocity and enzyme-substrate dissociation constant in the presence of an inhibitor. In the case of uncompetitive inhibition, $V'_{max} = V_{max}/\alpha$ and $K'_m = K_m/\alpha$ where

$$\alpha \quad = \quad 1 + [I] / K_i \qquad (8)$$

2.6.2.2.3 Linear mixed inhibition

In this type of reversible inhibition, a compound can interact with both the free enzyme and the enzyme-substrate complex at a site other than the active site:

$$\begin{array}{ccccc} & & K_m & & K_{cat} & \\ & E + S & \leftrightarrow & ES & \rightarrow & E + P \\ & + & & + & & \\ & I & & I & & \\ K_i & \updownarrow & & \updownarrow & \delta K_i & \\ & EI + S & \leftrightarrow & ESI & & \\ & & \delta K_m & & & \end{array}$$

This results in an apparent decrease in V_{max} and an apparent increase in K_m. The rate equation for the formation of product, the dissociation constants for enzyme-substrate (ES and ESI) and enzyme-inhibitor (EI and ESI) complexes, and the enzyme mass balance are, respectively,

$$v \quad = \quad K_{cat}[ES] \qquad (9)$$

$$K_m = [E][S]/[ES \quad \delta K_m = [EI][S]/[ESI] \quad K_i = [E][I]/[EI] \quad \delta K_i = [ES][I]/[ESI]$$

$$[E_T] \;=\; [E] + [ES] + [EI] + [ESI] \;=\; [E] + [E][S]/K_m + [E][I]/K_i + [E][S][I]/K_m\,\delta K_i$$

Normalization of the rate equation (9) by total enzyme concentration ($v/[E_T]$) and rearrangement results in the following expression for the velocity of an enzymatic reaction in the presence of a linear mixed type inhibitor, where $V_{max} = K_{cat}[E_T]$

$$\begin{aligned} v &= V'_{max}[S] / (K'_m + [S]) \\ &= (V_{max}/\beta)[S] / \{(\alpha/\beta)K_m + [S]\} \end{aligned} \qquad (10)$$

Where V'_{max} and K'_m correspond, respectively, to the apparent enzyme maximum velocity and apparent enzyme-substrate dissociation constant in the presence of an inhibitor. In the case of linear mixed inhibition, $V'_{max} = V_{max}/\beta$ and $K'_m = \alpha K_m$, where

$$\alpha = 1 + [I] / K_i \qquad (11)$$

and

$$\beta = 1 + [I] / \delta K_i \qquad (12)$$

2.7 Sorption of PCP and PAHs to humic substances

More and more aromatic pollutants come into the environment as agrochemicals or waste products from numerous syntheses. Most of these pollutants show unfavorable effect on flora and fauna. The pollutants which in major are from anthropogenous sources have their fates in environment. One of the primary fates of those aromatic pollutants in terrestrial and aquatic environments is sorption to natural organic matter. The strong association between pollutants and organic matter often limits passive remedial methods, bioremediation, and encourages persistence in the environment. Moreover, most of the aromatic pollutants exhibit toxicity even at low concentrations (Schwarzenbach, et al., 1993). Therefore, sorption mechanisms should be understood to improve risk assessment, fate and transport models, and remedial methods of aromatic pollutants.

The degree of sorption can be described in term of distribution coefficients or K_d, which is an organic chemical distributes between an environmental solid and aqueous phase at equilibrium. It is normally derived from the slope of sorption isotherm at the pollutant concentrations. The higher value of K_d shows the higher amount of sorption. The sorption isotherms can be linear or nonlinear depending on partitioning and adsorption mechanisms. The linear relationship equation is:

$$K_d = C_s * C_w^{-1} \qquad (13)$$

where C_s and C_w are the concentration of the pollution sorbed by solid phase (mg/kg) and dissolved in aqueous phase (m/L), respectively. Units of K_d can be L/kg, ml/g, or cm^3/g.

The pollutants of interest in this study were pentachlorophenol (PCP) and phenanthrene. As reviewed, PCP also showed the propensity to be sorbed into HS (Paolis and Kukkonen, 1997; Schellenberg, 1984; Peuravuori et al., 2001). 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). Several partition coefficients have been determined for PCP with different natural solids and organic solutes. For example, Peuravuori et al. (2001) determined sorption of PCP on dissolved aquatic humic matter for very wide concentration range from 60 ng PCP/L – 1 mg PCP/L). They proposed normalized sorption coefficients (log K_{oc}) of 3.19 at pH 5.5 ($K_{oc} = [C]_{oc} / [C]_{aqueous}$). Paolis and Kukkonen (1997) found the higher value for log K_{oc} for dissolved HA of 3.90 than for dissolved FA of 3.00 in PCP binding affinity. They also suggested that binding efficiency was pH dependent. Only the unionized form of PCP can interact with the humic materials. For example, at pH 5.0,

PCP had higher binding coefficients than at pH 8.0. However, log K_{oc} values of PCP are very large, even a value of 4.5 has been found by Schellenberg (1984). As stated by Peuravuori et al. (2001), these different values were depending on analytical methods, algorithms applied, the nature of humic matters, the acidity and ionic strengths of the solution, resulting in various mechanisms to operate simultaneously in the sorption process.

A main fate of nonvolatile, nonionic organic contaminants such as phenanthrene is sorption to soil or sediment organic matter (Schwarzenbach, et al., 1993). Salloum et al. (2002) reported log K_{oc} = 4.67 for phenanthrene sorbed by humic acid from black shale that contained inclusions of coal, sampled along the banks of the Blackmud Creek, located south of Edmonton, Alberta, Canada. Humic acids extracted from mature mushroom compost, peat from International Humic Substances Society (IHSS), and soil from the Ellerslie Research Station, located south of the University of Alberta, Edmonton, Canada exhibited log K_{oc} for phenanthrene sorption coefficient of 4.18, 4.15, and 4.43, respectively. Vacca et al. (2005) reported phenanthrene sorption coefficient indicated as K_p = 33 to dissolved Aldrich humic acid ($K_p = [C]_{sorbed} / [C]_{aqueous}$). They experimented on equilibrium dialysis method using 83 mg/L of phenanthrene and 20 mg/L of Aldrich humic acid. It is also suggested that the bulk or macroscopic sorbent characteristics, such as polarity or aromaticity, can be correlated to and account for differences in sorption behavior (Garbarini and Lion, 1986; Grathwohl, 1990; Xing et al., 1994). Contradictorily, some studies have indicated that aromaticity is not suitable to explain K_{oc} values (Chefetz et al., 2000; Salloum et al., 2001a,b, 2002). They claimed that aliphatic functionalities should also play an important role in sorption behavior of polycyclic aromatic hydrocarbons to humic substances.

2.8 HS and ligninolytic enzyme reaction studies

Since HS contain functional groups susceptible to enzyme degradation, their structures might be enzymatically altered after HS – enzymes incubation. HS characteristics analysis will be useful to investigate the reaction between HS and ligninolytic enzymes. The technique in the field of HA and FA analysis include size exclusion chromatography methods (Fuzzi and Zappoli, 1996; Zappoli et al., 1999; Krivácsy, et al., 2000) or spectroscopic methods such as diffuse reflectance infrared Fourier transform spectroscopy, UV or fluorescence spectroscopy (Havers et al., 1998; Zappoli et al., 1999; Kiss et al., 2003). UV-vis spectroscopic method would be used in this research because it is available and useful for characterization of HS. Nurmi and Tratnyek (2002) studied chemical properties of natural organic matter including soil, peat, river and river HA and FA using 10 mg C/L by UV-vis spectroscopic method. One of the absorbance used in the study was 465 nm. It was known to be useful in quantitative characterizing of HS. Visible light absorption at 465 nm wavelength represents degree of humification (Tan, 2003), especially in an initial humification stage (Dębska et al., 2002). This absorbance can imply to heterocyclic, aromatic, carboxylic, and monoester functional groups (Olk, 2006). In addition, the 465 nm wavelength is useful to quantify dissolved HS (Mazzuoli et al., 2003). Cohen et al. (1987) experimented on solubilization of leonardite coal by purified laccase from *Polyporus versicolor* by monitoring the increase in absorbance at 450 nm by spectrophotometer. They used the mixture of 30 mg of coal with 3 ml of the buffer-cell filtrate. They reported the failure to show any alterations in the structure of HS which had particle sizes > 105 μm. The smaller particle sizes were degraded faster by the enzyme.