CHAPTER IV

ROLE OF DISSOLVED HUMIC SUBSTANCES AND DISSOLVED ORGANIC MATTER ON DEGRADATION OF PHENANTHRENE BY CRUDE LIGNINOLYTIC ENZYMES FROM *AGROCYBE* SP. CU 43

4.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are omnipresent contaminants found in air, soil, freshwater, and marine environments (Cerniglia and Heitkamp, 1989). Their fate and distribution in the environment are of increasing interest because four or more aromatic rings compounds are recalcitrant, carcinogenic, mutagenic, or toxic and pose serious human health risk effect (Blumer, 1976). Phenanthrene is one of the most abundant PAHs in the environment and widely used as model PAHs (Carniglia, 1992). Due to its toxic and persistence, management of phenanthrene is necessary. High cost of abiotic method to remove PAHs in the environment leads to the increase use of biotic method by using microorganisms to decontamination and detoxication of PAHs (Wilson and Jones, 1993). The use of white rot fungi have been widely investigated for bioremediation, for examples, for effluent treatment from chemical industry (Knapp and Newby, 1999; Schliephake et al, 1993), cotton bleaching (Davis and Burns, 1990; Zhang et al., 1998; Zhang et al., 1999), dyes (Spadaro and Renganathan, 1994; Wang and Yu, 1998; Pointing et al, 2000), molasses wastewater (Fitzgibbon et al., 1995; Kumar et al., 1998), olive oil milling wastewater (Sayadi and Ellouz, 1992; Pérez et al., 1998), paper making and pulping effluents (Prasad and Gupta, 1997; Peralta-Zamora et al., 1998; Srinivasan and Murthy, 1999) have been widely investigated. The white rot fungi have been extensively used due to their extracellular enzyme activities such as lignin peroxidase, manganese peroxidase and laccase (Paszcynski and Crawford, 1995). White rot

Agrocybe sp. CU 43 from yanagni mushroom was first isolated by Chupungars (2008) at Chulalongkorn University, Thailand. It has previously reported to have high potential in degrading a various types of PAHs, including phenanthrene (Chupungars, 2008), which our model PAH by ligninolytic enzyme system. The fungi could degrade 99.2% of phenanthrene within 21 days (Chupungars, 2008). Therefore, crude enzyme from *Agrocybe* sp. CU 43 was chosen in this study due to its promising ability to degrade phenanthrene. However, in aquatic and terrestrial environment, the abilities of the ligninolytic enzymes might be altered due to the presence of humic substances (HS) and dissolved organic matter (DOM).

Humic substances (HS) are major organic compounds in soil and sediment (Schnitzer, 1978; Stevenson, 1994). They are significantly heterogeneous mixtures of macromolecules with high molecular weight. Due to their solubility, three humic fractions are extractable. Fulvic acid (FA) is soluble in both acid and basic solutions, and humic acid (HA) is soluble in base but insoluble in acid solutions. Humin is the soil/sediment organic matter fraction not extractable in alkaline solution (Stevenson, 1994). HS is a subset of DOM, and DOM additionally contains polysaccharides, including lignin, cellulose, and hemicellulose, proteins and amino acids, phenols and polyphenols, quinones, carbohydrates, and lipids (Tan, 2003). DOM was used in the experiment because we would like to determine that if other dissolved organics besides HS will interact with the enzymes. HS and DOM have been widely reported to influence the solubility, mobility and bioavailability of aromatic organic pollutants. However, among the researchers, there are different evidences for the roles of HS and DOM in mineralization and degradation for the aromatic pollutants.

One of the possible role of HS and DOM in aromatic pollutant degradation is they can compete for the oxidation of aromatic pollutants and therefore inhibit

transformation rate of the pollutant (Itoh et al., 2000). Due to the complex structure of HS and DOM, they comprise the numerous function groups, including carboxyls, alcoholic and phenolic hydroxyls, carbonyls, and methoxyls (Essington, 2004). Those functional groups could be substrates for nonspecific ligninolytic enzymes. For example, Zavarzina et al. (2004) investigated the inhibition effect of HA by *Panus tigrinus* laccase. They reported K_i ranged from 0.003 μg/mL for HA from peat soils to 0.025 μg/mL for HA from chernozems. Nevertheless, Holman et al. (2002), Bengtsson and Zerhouni (2003), Bogan and Sullivan (2003) reported the increase in mineralization and biodegradation of organic pollutants with HS addition.

Humic substances are able to react with white-rot ligninolytic enzymes such as manganese peroxidase and laccase (Yavmetdinov et al., 2003). The interaction of the enzymes with HS may lead to depolymerization of HS and their synthesis from monomeric precursors. These two processes can be dependent on the nature of HS (Zavarzina et al., 2004). For example, decolorization and decrease of HA's molecular weight and the formation of FA after incubation of the HA with *Trametes versicolor* were reported by Fakoussa and Frost (1999). Contradictorily, with the same culture, the formation of HA was investigated by Katase and Bollag (1991). Humic substances could either stimulate or inhibit enzyme activity dependent on their origin and characteristics (Clause and Filip, 1990). HA and its monomeric constituents either increase (Wang et al., 2002) 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.

A main fate of nonvolatile, nonionic organic contaminants such as phenanthrene is sorption to soil or sediment organic matter (Schwarzenbach, et al., 1993). A number of sorption studies have grown to progress the more understanding of pollutant behaviors in environmental fate, improving bioremediation technique. Salloum et al. (2002) reported log K_{oc} = 4.67 for phenanthrene sorbed by humic acid from black shale that contained inclusions of coal. Humic acids extracted from mature mushroom compost, peat from International Humic Substances Society (IHSS), and soil from the Ellerslie Research Station, University of Alberta, 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 Aldrich humic acid. K_p (Phenanthrene partition coefficient can be converted to K_{oc} following the equation: $K_p = K_{oc}.f_{oc}$, f_{oc} = fraction of organic carbon in HS). 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., 2001, 2002). They claimed that aliphatic functionalities should also play an important role in sorption behavior of polycyclic aromatic hydrocarbons to humic substances.

To date there has been no clear explanation for mechanisms of which how HS play a role in organic pollutant degradation. To identify the possible role of HS and DOM on enzymatic degradation rate of aromatic pollutants, degradation of phenanthrene, a model compound, by crude ligninolytic enzymes from *Agrocybe* sp. CU 43 was studied. Three hypotheses of HS and DOM's role were 1) HS and DOM

can deactivate enzymes 2) HS and DOM can compete with aromatic pollutants for enzyme 3) HS and DOM is inert and protects aromatic contaminant. The degradation kinetics and reversibility of the inhibition were studied at different concentrations and types of HS and DOM. Binding effect of HS and DOM on phenanthrene was also studied.

4.2 Materials and Methods

4.2.1 Materials

4.2.1.1 Humic substances

Commercial HS supplied by Fluka company and International Humic Substances Society (IHSS) were used in the research to promote the comparisons with other HS studies. Chemical and physical properties of experimental HS were shown in Table 4.1. AHA and LHA were dissolved in 0.5 M NaOH solution and adjusted its volume by distilled water. Final pH for the stock AHA and LHA were 8.27 and 8.61, respectively. SRFA and WFA were dissolved in distilled water. The stock fulvic acids had final pH of 3.19 and 3.29 for SRFA and WFA. 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; which affecting phenanthrene enzymatic degradation rate and sorption phenomena. HS could be one of the important factors that make us misestimate the transformation rates of pollutants in various soil types.

Table 4.1 Chemical and physical properties of particular humic substances

Humic substances	Sources	% Aromaticity	% Carboxyl	% C	% O	% N	Weight average 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.

4.2.1.2 Dissolved organic matter

4.2.1.2.1 Soil sample collection and preparation

Soil sample used in these experiments was from paddy field in Na-Klang District, Nongbualumphu province, Thailand. Surface soil was collected from 10 – 15 cm depth. The paddy field soil was selected in the experiment because it was widespread and could be a soil representative in Thailand. The sample was air-dried and homogenized by sieving (< 2 mm). Then, chemical and physical properties of the soils were analyzed.

4.2.1.2.2 Determination of soil characteristics

The soil samples were analyzed for physiochemical properties including soil texture (sieve analysis), pH (soil:water, 1:1), cation exchange capacity (CEC)

(Ammonium saturation and distillation method) and total organic carbon by TOC analyzer (Analytik Jena, Multi N/C 2100). The paddy field soil contained 21.8% sand, 25.8% silt, and 52.4% clay. It had pH of 5.20, CEC of 14.6 cmol/kg and TOC of 1.52 %. The soil is classified as a USDA soil taxonomy order of Ultisols as referenced by United States Department of Agriculture, Natural Resources Conservation Service (http://soils.usda.gov/technical/classification/taxonomy/, 2008). The Ultisol soil is defined as mineral soils which contain no calcareous material anywhere within the soil, have less than 10% weatherable minerals in the top soil, and have less than the 35% base saturation throughout the soil. Typically Ultisols are red to yellow in color, due to high accumulation of iron oxide, and quite acidic (http://en.wikipedia.org/wiki/Ultisol, 2008).

4.2.1.2.3 Preparation of dissolved organic matter (DOM)

The soil was stored at -20°C and thawed at 4°C overnight to increase the release of DOM (Raber and Kögel-Knabner, 1997). Then, the soil was extracted for 2 h with deionized water pH 7.0 using a soil:water ratio of 1:2 (by weight). The suspension was centrifuged for 30 min at 3600 g (Sorvall® Biofuge Stratos) and filtered through a 0.45 µm micro fiber filter (GF/C, Whatman®, Schleicher & Schuell). The solution was analyzed for DOM by TOC analyzer (Analytik Jena, Multi N/C 2100). The soil comprised 63.8 ± 0.07 mg/L DOM. The extract was stored at 4°C in the dark no longer than 5 days.

4.2.1.3 Ligninolytic enzymes

4.2.1.3.1 Fungal cultivation and enzyme induction

Agrocybe sp. CU 43 was cultivated and induced for ligninolytic enzymes following Chupungars (2008). Inoculums were prepared by growing the fungus on

malt extract agar for 10 days at room temperature. Then, 5 – 6 pieces of 1 x 1 cm agar containing mycelia were transferred to 100 mL malt extract broth in a 500 mL Erlenmeyer flask and incubated at 28°C on an orbital mixer incubator (Ratek) at 120 rpm for 14 days. The cultured broth was transferred to a sterilized centrifuge tube and centrifuged at 10000g for 10 minutes and washed twice with sterilized deionized water. The supernatant was decanted and the fungal pellet was weighed. Ten grams of the pellet was transferred to N-limiting medium 100 mL in 500 mL Erlenmeyer flask and incubated at 28°C on an orbital mixer incubator at 120 rpm for 7 days. Then, 100 mg/L of phenanthrene was added and incubated at the same condition. At the cultivated time, 5 mL of the broth was centrifuged (Sorvall® Biofuge Stratos) at 10000g for 20 minutes. The supernatant which contained ligninolytic enzymes and the remaining phenanthrene was tested for their activities using catechol assay.

4.2.1.4 Chemicals

Phenanthrene was purchased from Aldrich Company, USA. Stock solution containing 1 g/L of phenanthrene was prepared in hexane to obtain the absolute dissolution. Hexane, sodium acetate, acetic acid and sodium hydroxide were purchased from Merck or Sigma Aldrich and were of the highest chemical purity available.

4.2.2 Methods

4.2.2.1 Enzyme activities

Enzyme reaction contained 10 - 40 mg/L HS or DOM and 6 U laccase using 200 mM sodium acetate buffer (pH 5) to adjust its final volume to 3 mL and incubated at 28°C, 200 rpm. The samples were sacrificed every 24 h for 96 h. Then,

laccase activity was measured by adding 100 mM catechol to the sample and observed absorbance change at 440 nm for 1 minute by UV-vis spectrophotometer (Specord 40, Analytik Jena AG). One unit of laccase activity is defined as that which caused a change in absorbance of 1.0 OD/min/mL (Ullah et al., 2000). The experiments were performed in triplication.

4.2.2.2 Sorption and desorption of phenanthrene to HS and DOM

Sorption experiment was performed by equilibrium dialysis method. Firstly, equilibrium time needed to be investigated. Dialysis tubing was prepared from Spectra/Por® Biotech Cellulose Ester (CE) dialysis membranes MWCO 500 (Spectrum Laboratories Inc.) and filled with 3 mL of 15 mg/L of LHA solution. The tubes were placed in a 500 mL-beaker containing 200 mL of 200 mM sodium acetate buffer pH 5.0 and 20 mg/L of phenanthrene. LHA was selected for equilibrium time experiment because it occupied the highest percent aromaticity and molecular weight. These characteristics are believed to be susceptible to binding capacities of aromatic pollutants. LHA 15 mg/L and phenanthrene 20 mg/L were the highest concentration used for enzyme kinetics experiment. Phenanthrene concentration was about 20 times higher than its water solubility. However, this HS concentration represented the upper range of HS concentrations previously reported to enhance the solubility of hydrophobic compounds (Chien and Bleam, 1997; Chien et al., 1997; Vacca et al., 2005). To avoid photolysis, the beakers were wrapped with aluminum foil. Then, they were closed with wrapping film (polyvinylchloride cling film, M Wrap, MMP Packaging Group Co., Ltd) and placed on magnetic stirrer. Aliquots of the solution inside the tubing were removed for phenanthrene analysis every 24 h for 168 h. It was found that a 4-day period was enough for the equilibrium for phenanthrene-LHA

binding. To study the binding capabilities of phenanthrene to HS or DOM, the same experiment was carried out, except using AHA, SRFA, WFA, and DOM.

Phenanthrene desorbing from the HS and DOM were also determined by equilibrium dialysis. The dialysis tubing contained 3 mL of the mixture of 15 mg/L of HS or DOM spiked with the phenanthrene at equilibrium concentration. The tubing was placed in 17 mL of fresh 200 mM sodium acetate buffer pH 5.0 in 22 mL test tubes. The samples were shaken at 200 rpm on the shaker. Then, free phenanthrene in the tubes was extracted and analyzed by GC-FID at 48 h. This 48 h was the time to allow phenanthrene desorbed from AHA (Vacca et al., 2005).

4.2.2.3 Enzyme kinetics

4.2.2.3.1 Kinetics of phenanthrene and crude fungal ligninolytic enzyme

Crude fungal enzyme 6 U was incubated with the 10, 15, 20 mg/L of phenanthrene concentrations for a set period of time over which the reaction was still linear. A set of these phenanthrene concentrations was intended to give the initial reaction of phenanthrene degradation. One of the given HS and DOM at the concentration of either 10 or 15 mg/L was added as a competitive inhibitor. The concentrations of HS and DOM were preliminary experimented to lower phenanthrene degradation rate linearly. The same experiment was carried out, then, the results of K_m and V_{max} of the conditions with and without HS and DOM were compared.

4.2.2.4 Reaction of HS and DOM with crude fungal ligninolytic enzyme

This experiment was to test whether HS and DOM could be substrates for ligninolytic enzyme. Various concentrations of HS and DOM ranging from 10 – 40

mg/L were mixed with 6 U of the enzyme. One unit of enzyme was specified by the oxidation of 100 mM catechol where its absorbance at 440 nm changes in 1 minute. Sodium acetate buffer (200 mM) pH 5.0 was added to adjust their volume to 3 mL. The samples were shaken at 200 rpm at 28◦C until their light absorptions at 465 nm by UV-vis spectrophotometer were performed every 24 h for 96 h. Ligninolytic enzymes exhibited their absorption at 465 nm of 0.065 ± 0.00.

4.2.2.5 Phenanthrene determination

The samples were extracted by hexane and dewatered with sodium sulfate. Then, phenanthrene concentrations were analyzed by GC-FID. The GC equipped with HP 5 MS column (30 m x 0.25 mm id x 0.25 μm) and set the condition of carrier, helium 33 cm/sec constant flow; oven, 100°C for 0 min, 100 - 200°C at 6°C/min for 2 min, 200 – 250°C at 50°C/min for 0 min; injector, 5 μL splitless 250°C, retention time, 12.3 min. Percent recovery by this procedure was 86 – 103%.

4.3 Results and discussion

4.3.1 Phenanthrene degradation rate

Initial phenanthrene degradation rates by crude fungal ligninolytic enzyme in the absence and presence of HS and DOM were shown in Figure 4.1. The slower phenanthrene degradation rate in HS and DOM addition samples were found, validating that HS and DOM could decrease phenanthrene degradation rate in the system of interest. Since the degradation rate of phenanthrene was fitted to a linear line, a pseudo first order reaction was assumed in the system. The pseudo first order reaction can be represented as:

$$dC/dt = k'[C]$$

where k′ is the true rate constant, k, multiplied by HS concentration (mg/L) and [C] is the phenanthrene concentration [M]. The k′ values are concluded in Table 4.2.

Table 4.2 k′ degradation rate constants (hr^{-1})

HS concentration	0 mg/L	10 mg/L	15 mg/L
Sample			
Phenanthrene	1.39×10^{-3}	Na	na
Phenanthrene + AHA	na	1.05×10^{-3}	1.09×10^{-3}
Phenanthrene + LHA	na	9.63×10^{-4}	7.97×10^{-4}
Phenanthrene + SRFA	na	1.27×10^{-3}	1.25×10^{-3}
Phenanthrene + WFA	na	1.13×10^{-3}	1.12×10^{-3}
Phenanthrene + DOM	na	6.79×10^{-4}	7.10×10^{-4}

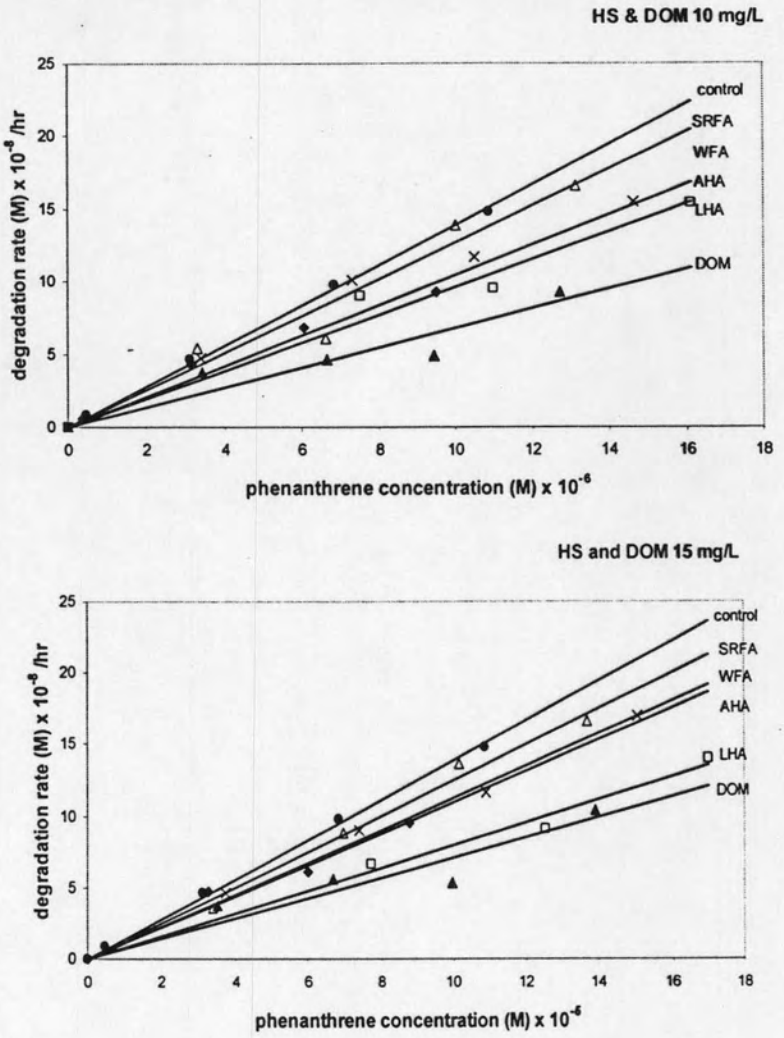

Figure 4.1 Phenanthrene degradation rate when HS or DOM 10 and 15 mg/L were added. Symbols represent HS or DOM, ●: control (phenanthrene only), ♦: AHA, □: LHA, Δ: SRFA, ×: WFA, ▲: DOM

4.3.2 Enzyme activity for HS and DOM with crude fungal ligninolytic enzymes

Enzyme activity was tested by using 100 mM catechol as a substrate and measured their absorbance change at 440 nm in 1 minute by UV-vis spectrophotometer every 24 h for 96 h. It was found that enzyme activities were not inactivated by HS and DOM at various concentrations. However, the activities were lower with WFA after 48 h. In fact, enzyme activity of 40 mg/L of DOM could not be measured by catechol assay due to their turbidity (data not shown). Percent enzyme activity was shown in figure 4.2. Therefore, we could invalidate the theory of which HS could deactivate enzymes.

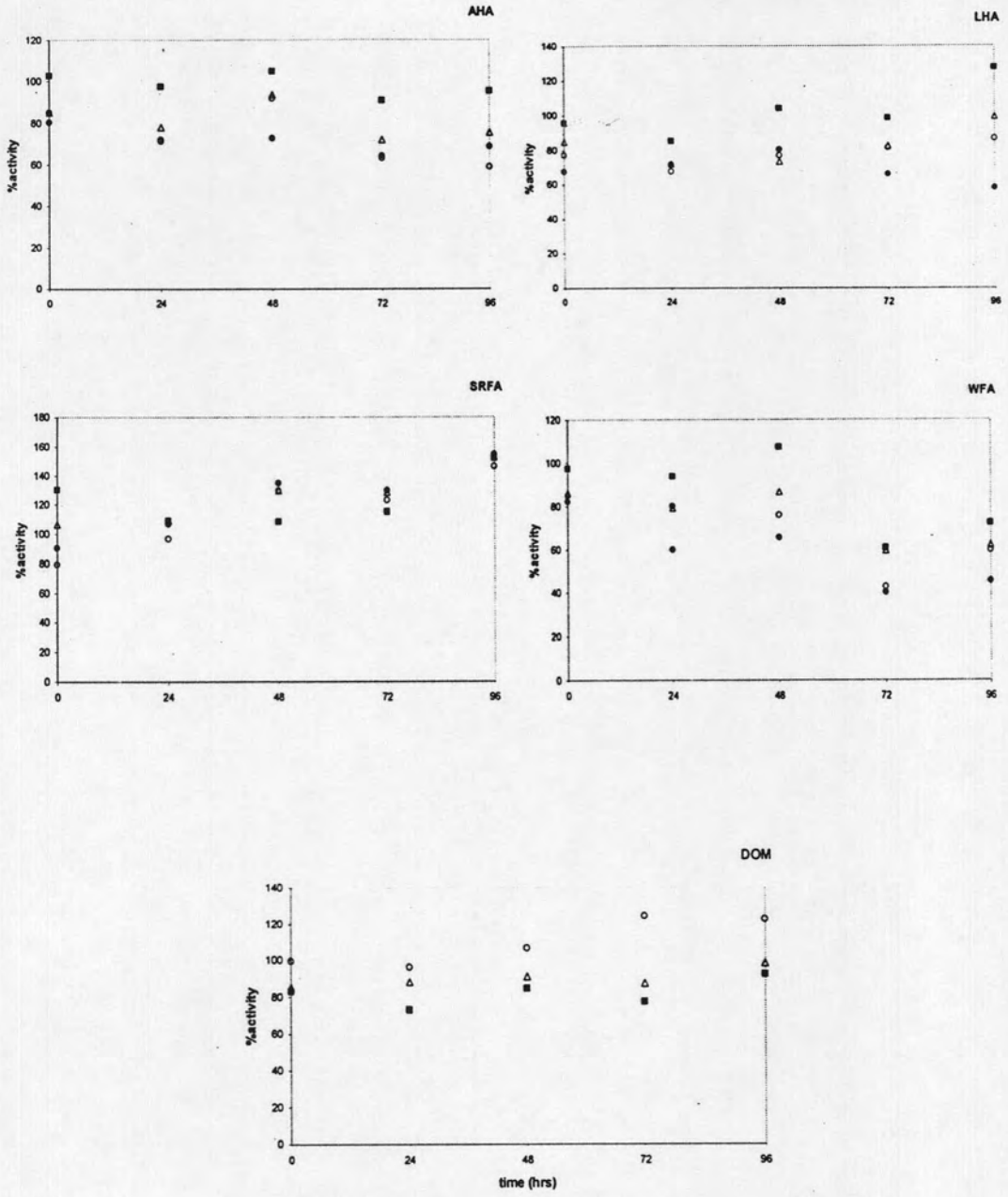

Figure 4.2 Percent enzyme activity for each HS and DOM. Activity of crude fungal ligninolytic enzyme in oxidation of catechol without HS and DOM is 100%. Symbols represent HS and DOM concentration, ■: 10 mg/L, Δ: 20 mg/L, ○: 30 mg/L, ●: 40 mg/L

4.3.3 Phenanthrene binding studies

After 96 h equilibrium time for allowing phenanthrene diffusion across dialysis membrane, phenanthrene-HS complex was collected. Phenanthrene concentration (free and HS-bound phenanthrene) per mg of HS inside the dialysis tubing was determined and showed in Table 4.3. It was found that phenanthrene partition coefficient (K_p) to HS was SRFA < WFA < AHA < LHA < DOM. The result agreed with our previous result using pentachlorophenol and the same HS. The group of aquatic fulvic acids (WFA, SRFA) bound less strongly than that of terrestrial humic acids (AHA, LHA) and DOM. Desorption experiment showed very low concentration (< 0.05 mg/L) of phenanthrene that could desorb out from the bound compound.

Table 4.3 Phenanthrene partition coefficients to HS and DOM

Humic substances	K_p (mol/kg)
AHA	0.64 ± 0.04
LHA	0.69 ± 0.01
SRFA	0.46 ± 0.00
WFA	0.48 ± 0.00
DOM	0.71 ± 0.02

Our result also supported Gourlay et al. (2003) and Bejarano et al. (2005) who reported that hydrophobic organic chemicals could interact with DOM, decreasing the contaminants' bioavailability. Pan et al. (2007) explains that DOM could associate with the hydrophobic organic chemicals and affect the environment fate of the chemicals. As claimed by Alexander (1995) and Nam and Alexander (1998), those organic pollutants associated with DOM could be an available substrate for microorganisms when they are dissolved in solution phase. Nature and extent of HS

could be important in controlling the association of HS and phenanthrene, affecting enzymatic degradation rate. Clapp et al. (1997) noted that the origin of HS played a key role in binding, and suggested that the extent of coiling of HA polymer strands is an important factor in their complex formation with nonionic organics.

We found the direct relationship between physicochemical properties of the HS and the degree of phenanthrene binding as shown in Figure 4.3. Strong correlation between binding capacities and aromaticity in HS was observed ($r^2 = 0.81$, Figure 4.3 (a)). The high correlation with %aromaticity indicates a specific interaction mechanism involving aromatic functional groups, e.g. pi-pi interactions. The same trend was also reported for Chin (1997), Uhle (1999), Perminova (1999), and Gadad (2007). It was possible that polarizability of HS is increased for the more aromatic HS (Gautheir, 1987; Chin, 1997). An increase in the polarizability of the HS could cause an increase in van der waals interactions between phenanthrene and HS. Our result agreed with that binding coefficient is dependent on aromatic nature of HS. However, poor relationship between binding coefficient and molecular weight of HS were found ($r^2 = 0.28$, Figure 4.3 (b)). The lack of correlation with weight averaged MW suggests that non-specific mechanisms such as hydrophobic interactions are not as important as specific interactions such as pi-pi bonding between phenanthrene and dissolved HS. MacCarthy (1988) explained a weak correlation between binding coefficient and molecular weight of HS that it might be due to heterogeneity and complex structure of HS.

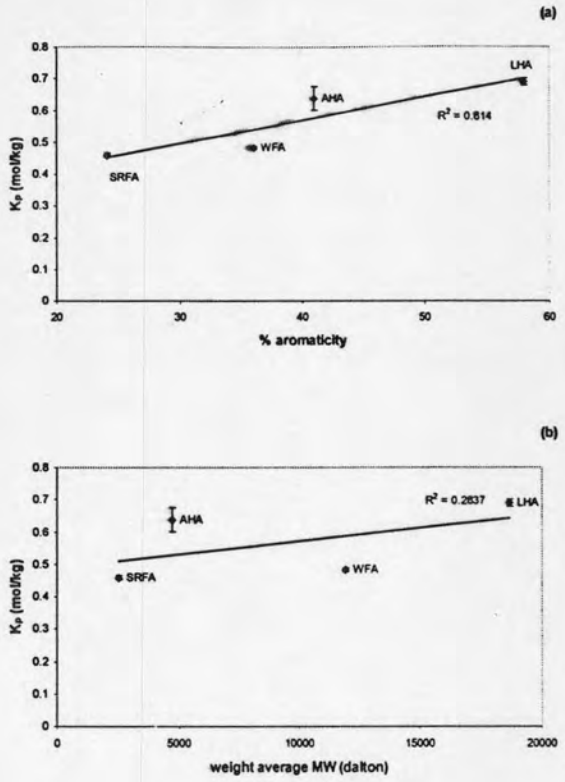

Figure 4.3 Linear relationship between binding coefficient and (a) % aromaticity (r^2 = 0.81), (b) molecular weight (r^2 = 0.28)

4.3.4 Inhibitory effect of HS and DOM on phenanthrene degradation by crude fungal ligninolytic enzymes.

The reaction of phenanthrene with 6 U of ligninolytic enzyme showed Michaelis-Menten kinetics in the substrate range of 0 – 20 mg/L, with a K_m value of 0.20 ± 0.04 mM and a V_{max} of 0.01 ± 0.00 μM/min. The samples with 10 and 15 mg/L of HS were converted in terms of dissolved organic carbon (DOC) concentration (DOC concentration (mg/L) = DOM concentration (mg/L) x % organic carbon / total mass of HS) so that K_m and V_{max} of HS and DOM could be calculated. K_m of AHA, SRFA, WFA, V_{max} values were not significantly different (Table 4.4). However, the results showed the higher K_m and lower V_{max} for increasing concentration of LHA and

lower K_m and V_{max} for increasing concentration of DOM extracted from paddy field soil samples. The difference in K_m and V_{max} when HS and DOM were added might be because they could act as competitive substrate for phenanthrene, or binding phenomena of HS-phenanthrene complex could occur in the system. The two hypotheses would be proven afterward.

Table 4.4 K_m and V_{max} of ligninolytic enzyme for phenanthrene with and without HS and DOM addition

HS	[DOC] (mg/L)	K_m (mM)	V_{max} (μM/min)
Control (Phenanthrene)	-	0.20 ± 0.04	0.01 ± 0.00
AHA	6.53	0.43 ± 0.02	0.01 ± 0.00
	9.80	0.55 ± 0.00	0.01 ± 0.00
LHA	6.38	0.22 ± 0.03	0.005 ± 0.001
	9.57	0.23 ± 0.02	0.003 ± 0.000
SRFA	5.30	0.36 ± 0.03	0.01 ± 0.00
	7.95	0.41 ± 0.27	0.01 ± 0.00
WFA	5.36	0.41 ± 0.07	0.01 ± 0.00
	7.95	0.56 ± 0.09	0.01 ± 0.00
DOM	10	0.09 ± 0.01	0.002 ± 0.000
	15	0.08 ± 0.02	0.002 ± 0.000

4.3.5 Inhibitory effect of HS on crude fungal ligninolytic enzyme

An inhibitory effect of HS was competitive type for AHA, SRFA, and WFA due to an increase in K_m and the same V_{max} while HS concentration was increased. This equation, $K_i = I / [(K'_m / K_m) - 1]$, is used to calculate K_i. The competitive inhibition means an inhibitor competes with an enzyme's substrate for binding to the active site. However, for LHA, increase K_m and decrease V_{max} when increasing HS concentration, its reaction could be linear mixed inhibition type. In this type of

reversible inhibition, the inhibitor may interact with both the free enzyme and the enzyme – substrate complex at a site other than the active site: (Marangoni, 2003)

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

Mix inhibition needs δK_i for enzyme-inhibitor constant value by calculated from

$$\beta = V_{max} / V'_{max}$$

$$\alpha / \beta = K'_m / K_m$$

$$K_i = [I] / \alpha - 1$$

$$\delta = [I] / (\beta - 1) K_i$$

DOM could exhibit uncompetitive inhibitor type because as DOM concentration decrease, K_m and V_{max} were decreased. HS could interact with the enzyme-substrate complex at a site other than the active site,

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

$K_i = [I] / (\alpha - 1)$, when $V'_{max} = V_{max}/\alpha$, $K'_m = K_m/\alpha$. V'_{max} and K'_m are the values with inhibitor. [I] is an inhibitor concentration. Lineweaver-Burke plots of ligninolytic enzyme for phenanthrene when HS and DOM were added as inhibitors were shown in Figure 4.4. K_i for each HS can be concluded in Table 4.5. Among HS group, K_i for DOM < LHA < AHA < WFA < SRFA was found. The inhibitory effect of HS was in

accord to the results of Gianfreda and Bollag (1994) and Zavarzina et al. (2004). They reported a linear relationship between the organic matter content and its inhibitory effect on activities of laccase. The inhibitory properties were shown for other enzymes incubated with HS such as lignin peroxidase (Wondrack, et al., 1989), pronase, trypsin and carboxypeptidase (Ladd and Butler, 1971). Because binding mechanism was proven to control phenanthrene degradation rate, the inhibitory effect of HS confirmed that only free phenanthrene could be substrate for enzyme, affecting degradation rate of phenanthrene in our system.

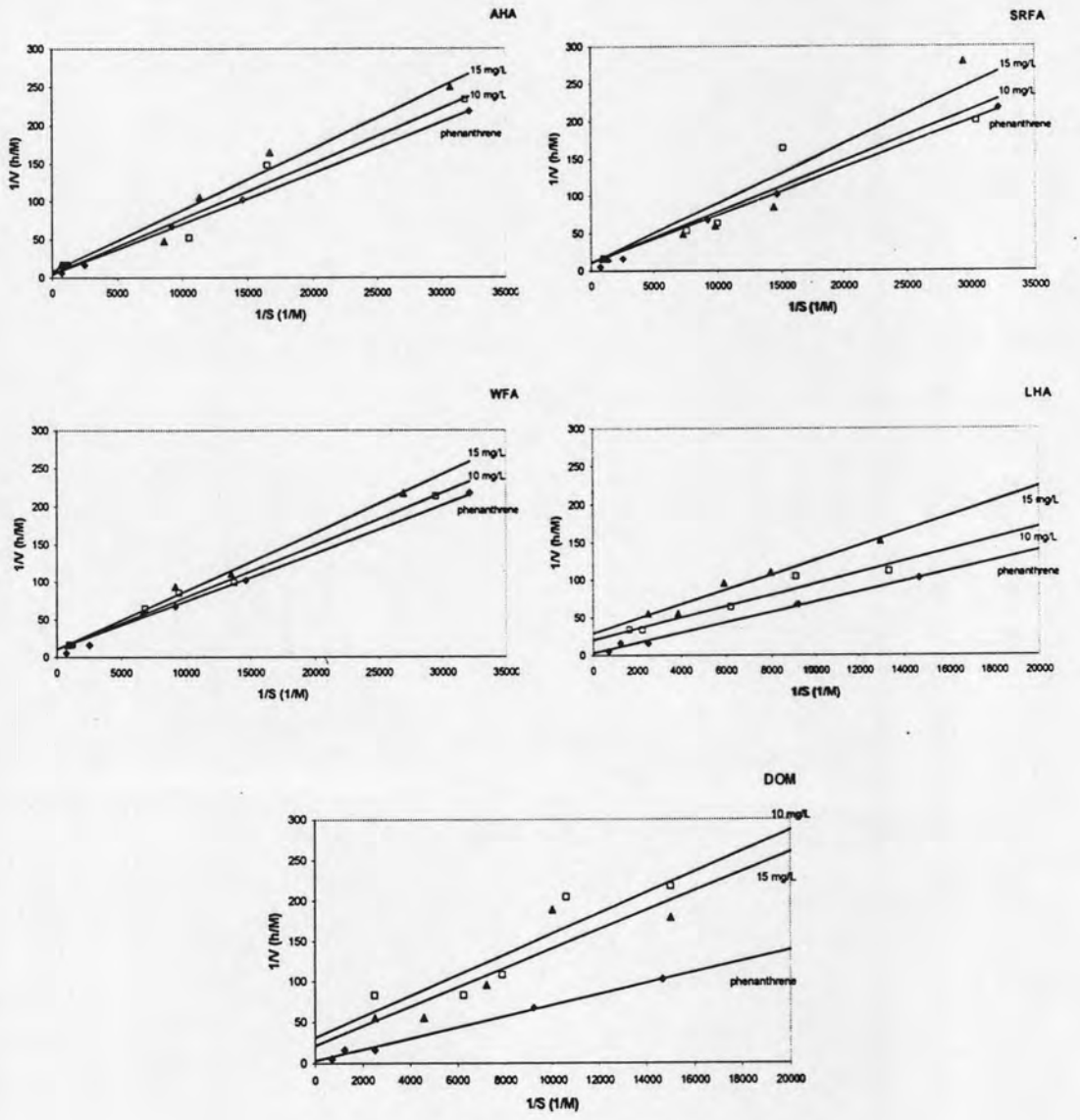

Figure 4.4 Lineweaver-Burke plot of ligninolytic enzyme for phenanthrene when HS and DOM were added as inhibitors

Table 4.5 K_i for each HS

Humic substances	K_i (mg/L)*
DOM	5.11
LHA	8.63
AHA	9.35
WFA	12.39
SRFA	16.81

*: AHA, SRFA, WFA used competitive inhibition model
LHA used linear mix inhibition model
DOM used uncompetitive model

4.3.6 Relationship between K_i and physical properties of HS

The inhibitory effect of HS had a strong relationship with % aromaticity (r^2 = 0.81) but poor correlation with molecular weight (r^2 = 0.39) (Figure 4.5). This suggested phenanthrene interaction with HS in addition to HS acting as a substrate. We proposed that aromatic group was an important factor in inhibitory effect of HS, including AHA, LHA, SRFA, and WFA, and DOM in the systems containing ligninolytic enzyme and phenanthrene. Zavarzina et al. (2004) reported the more hydrophobic HA were stronger inhibitors. Their early work of Zavarzina et al. (2002) also suggested that the hydrophobicity of HA may be due to the presence of aromatic structures (e.g., aromatic rings). This was correlated to our results that higher aromatic HS occupied stronger inhibitors. Moreover, Zavarzina (2004) suggested that MW of HS would not affect inhibitory effect as significant as those hydrophobic properties.

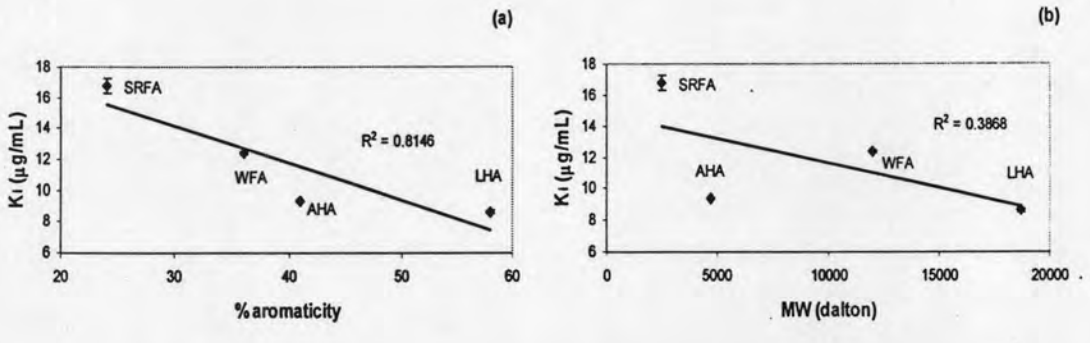

Figure 4.5 Relationship between K_i and (a) %aromaticity, (b) molecular weight

4.3.7 HS and DOM degradation by crude fungal ligninolytic enzymes

The objective of this section was to prove whether HS could act as a competitive substrate. The analysis of HS using UV-vis spectrophotometer at 465 nm wavelength was used. The 465 nm – absorbance could imply the heterocyclic, aromatic, carboxylic, and monoester functional groups of HS (Olk, 2006) and is useful in dissolved HS quantification (Mazzuoli et al., 2003). Unlike the reaction of HS and purified laccase enzyme from our previous experiment, the reaction of either HS or DOM with crude fungal ligninolytic enzyme showed the increase in 465 nm absorption. Yaropolov et al. (1994) explained that during lignin degradation by laccase two reactions, polymerization and depolymerization, can occur depending on initial molecular weight distribution in lignin preparations: polymer degradation with formation of low molecular weight products or condensation to form high molecular weight fractions indicative of existence of condensation – depolymerization equilibrium. In lignin degradation, ligninolytic enzyme is responsible for its demethylation, cleavage of $C_\alpha - C_\beta$ and alkyl – aryl bonds of phenolic substructures, and side-chain elimination. Condensation occurs due to spontaneous polymerization of free radicals, formed as a result of oxidation of hydroxyl groups in the presence of molecular oxygen (Reinhammer, 1984; Thurston, 1994). Absorbances of HS with purified laccase, and either HS or DOM with crude fungal enzyme were shown in Table 4.6.

Table 4.6 Absorbance at 465 nm of 40 mg/L of HS with laccase and crude fungal ligninolytic enzymes at 0 and 96 hours

Time (hours)	0	96
With laccase*		
AHA	0.5477 ± 0.0042	0.5218 ± 0.0088
LHA	0.7042 ± 0.0003	0.6866 ± 0.0034
SRFA	0.4817 ± 0.004	0.4372 ± 0.0254
WFA	0.5253 ± 0.0065	0.4828 ± 0.0130
With ligninolytic enzyme		
AHA	0.2450 ± 0.0025	0.2627 ± 0.0024
LHA	0.3221 ± 0.0070	0.3372 ± 0.0014
SRFA	0.0771 ± 0.0015	0.0811 ± 0.0025
WFA	0.1127 ± 0.0021	0.1178 ± 0.0019
DOM	0.5245 ± 0.0038	1.4421 ± 0.0177

*: as results from previous study

4.3.8 Effects of HS and DOM on phenanthrene degradation rate

Hypothesis stated that HS can deactivate enzymes was invalidated since our results showed that the enzymes still active with HS incubation. We found that HS could exhibit inhibitory effect on phenanthrene degradation and/or bind to phenanthrene. In this section we need to clarify by which mechanism could take place, influencing phenanthrene enzymatic degradation rate. Only freely dissolved phenanthrene is degraded by ligninolytic enzymes is assumed. The assumption could be proven by comparison of the experiment degradation rate and calculated degradation rate. The calculations of 1) the experimental degradation rates of phenanthrene in the presence of HS (k'[C]), 2) the calculated concentration of freely dissolved phenanthrene (assume phenanthrene concentration of 20 mg/L and using K_p for calculation), and 3) the calculated expected phenanthrene degradation rate for 20 mg/L of phenanthrene at 24 hr initial reaction were made. If this ratio = 1, it means only freely dissolved phenanthrene is degraded by ligninolytic enzyme. If the ratio <

1, it indicates that the degradation of HS by ligninolytic enzyme affects the overall phenanthrene degradation rate. If the ratio > 1, it suggests that phenanthrene bound to HS is available to some extent for enzyme degradation; either HS – associated phenanthrene is rapidly released or HS – associated phenanthrene is accessible to the enzyme. The experimental and calculated phenanthrene degradation rate constants were shown in Table 4.7 (See supplemental data for detailed calculation).

We found that both HS and DOM provide experimental degradation rate and calculate degradation rate ratio less than 1. It could imply that the phenanthrene degradation by ligninolytic enzyme was slower than expected and might be due to HS or DOM could act as another substrate and compete with phenanthrene for the enzyme. Therefore, we would like to propose the possible mechanism of HS and DOM influencing phenanthrene enzymatic degradation rate by ligninolytic enzyme. Firstly, HS and DOM could sorb phenanthrene and protect phenanthrene from enzymatic degradation. Then, freely dissolved phenanthrene would be degraded while HS and DOM could compete with phenanthrene for enzyme degradation. We also discover that characteristics of HS and DOM could be important in controlling the phenanthrene enzymatic degradation mechanisms. Strong relationship between %aromaticity and phenanthrene – HS / DOM binding suggests us to develop a predictive and quantitative model for enzyme-activated degradation. For example, since phenanthrene sorbs to HS / DOM, and this sorption is a function of aromaticity, two parameters important to know regarding enzymatic degradation are the K_{ow} of the substrate (the higher the K_{ow}, the greater the sorption to HS, and the lower the enzymatic accessibility), and the %aromaticity of HS (the more aromatic functional groups, the more available sites for pi-pi interactions with aromatic substrates such as phenanthrene).

Table 4.7 Experimental and calculated phenanthrene degradation rate constants

Sample	Experimental phenanthrene degradation rate constant (k'[C]) (M/hr) (A)	Calculated freely dissolved phenanthrene concentration (M) (B)	Calculated phenanthrene degradation rate considering only freely dissolved phenanthrene (M/hr) (C)	Ratio (A)/(C)
Phenanthrene	1.56×10^{-7}	na	na	na
+ AHA 15 mg/L	1.22×10^{-7}	1.02×10^{-4}	1.06×10^{-6}	0.11
+ LHA 15 mg/L	8.92×10^{-8}	1.01×10^{-4}	1.05×10^{-6}	0.08
+ SRFA 15 mg/L	1.40×10^{-7}	1.05×10^{-4}	1.09×10^{-6}	0.13
+ WFA 15 mg/L	1.25×10^{-7}	1.04×10^{-4}	1.08×10^{-6}	0.11
+ DOM 15 mg/L	7.95×10^{-8}	1.01×10^{-4}	1.05×10^{-6}	0.07

Conclusion

1. Humic substances are dominant organic components in soil and sediments. They can control microbial degradation of organic pollutants associated in them. We found that if phenanthrene entered the environment included these HS, they were capable to be sorbed, decreasing their bioavailability, while they were not inactivate ligninolytic enzyme.

2. We suggest the model of which, firstly, sorption phenomena would take a responsibility in controlling the mechanism to allow free phenanthrene available for enzymatic degradation. Then, our enzyme kinetics experiment found that HS could be competitive substrates either by competitive, linear mixed inhibitor, or uncompetitive models. The slower rate of phenanthrene degradation was due to the sorption of the contaminant to HS and inhibitory effect of HS due to the ratio of less than 1 between experimental degradation rate and calculated degradation rate considering only unbound phenanthrene.

3. % aromaticity of HS is an important factor to control association of HS and phenanthrene, and inhibition effect, therefore, affecting the enzymatic degradation rate.

4. We would like to underline that HS and DOM which are complex macromolecules and predominant in environment must be taken into account in bioremediation strategies. In application of wastewater treatment, conditions and species or strains should be well studied to specific problems. HS and DOM are one of other important factors that must be concerned in the treatment process. Wastewater DOM is highly heterogeneous in size and chemical composition. The molecule masses of the DOM range from less than 500 to more than 5000 Da (Imai et al., 2002). And it is composed of a mixture of humic materials, polysaccharides, polyphenols, proteins, lipids, and heterogeneous molecules. Determination of DOM chemical structure is difficult due to of this complex composition. We proposed the model of which binding of phenanthrene-HS or phenanthrene-DOM could influence enzymatic degradation rate of the pollutant. Since sorptive capability experiment of the pollutant to HS and DOM are more simply determined than HS or DOM structure characterization, the preliminary study of binding potential of hydrophobic organic pollutant and HS or DOM is essential for better understanding of enzymatically degradation of the pollutants.

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