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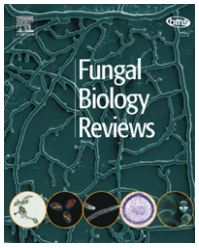

Review

Degradation and transformation of humic substances by saprotrophic fungi: processes and mechanisms

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ABSTRACT

Humic substances represent the main carbon reservoir in the biosphere, estimated at 1600×10^{15} g C. Due to their crucial role in reductive and oxidative reactions, sorption, complexation and transport of pollutants, minerals and trace elements, sustaining plant growth, soil structure and formation, and control of the biogeochemistry of organic carbon in the global ecosystem, humic substances are extremely important to environmental processes. Saprotrophic fungi active in the decomposition process of humic substances include mainly ascomycetes and basidiomycetes, which are both common in the upper layers of soils. White rot and litter decomposing fungi are the most important organisms in the degradation and mineralization of refractory organic matter (OM), whereas ascomycetes are mainly involved in the modification and polymerization of humic substances. The mechanisms of degradation probably involve mainly a variety of non specific oxidizing enzymes. This review provides an overview of the subject, while bridging two main disciplines: soil OM chemistry and fungal microbiology. It is aimed to highlight problems, unsolved questions and hypotheses.

1. Humic substances: background

Humic substances are natural non-living organic substances that are ubiquitous in the environment, both aquatic and terrestrial: they are found in sediments, peat, lignites, brown coal, sewage, composts and other deposits. Soil organic matter (SOM) represents the main carbon reservoir in the biosphere, estimated at 1600×10^{15} g C. There are about 1000×10^{15} g C in marine sediments and around 700×10^{15} g C are dissolved in seawater. These amounts are greater than in all land plants (600×10^{15} g C) and marine organisms (3×10^{15} g C) combined (Hedges *et al.* 2000). Thus, elucidating the role of microorganisms in the transformation and degradation of these compounds is crucial to an understanding of the global carbon cycle. However, the details of the processes and the role of fungi are far from being understood, differ from one fungus to another, change in diverse environments and depend on the substrate.

Humic substances are extremely important environmentally due to their crucial role in reductive and oxidative reactions (Bradley *et al.* 1998; Coates *et al.* 1998), sorption, complexation and transport of pollutants, minerals and trace

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elements (Kaschl et al. 2002; Simpson et al. 2003), sustaining plant growth (Chen et al. 2000), soil structure and control of the biogeochemistry of organic carbon in the global ecosystem (Stevenson 1994). Humic substances are formed by secondary synthesis reactions (humification) during the decay process and transformation of biomolecules originating from plants and other dead organisms. Lignin and its transformation products, as well as polyphenols, melanin, cutin, proteins and other derived polymers, are important building blocks in this process (Stevenson 1994).

In nature, humic substances (mainly humic acids and humin) are extremely resistant to biodegradation. Their half-decay time ($t_{1/2}$) can amount to thousands of years. For example, ^{14}C dating has shown that soil organic matter originating from the North American Great Plains or from volcanic soil environments is in the order of hundreds or thousands of years old. The stability of humic substances decreases with depth and is highly influenced by soil minerals (Paul et al. 1997; Torn et al. 1997). On pine-forest floors, for example, these numbers are much lower, with the $t_{1/2}$ in this environment at 5.1 y for the humic acid fraction and 3.9 y for the bulk litter. Other studies suggest that physical protection mechanisms are the most significant factors controlling the stability of OM (Mikutta et al. 2006; Von Lutzow et al. 2006).

In this review we describe and discuss the role of fungi in the turnover of humic substances.

2. Humic substance properties and structure

Based on their solubility in acids and alkalis, humic substances can be divided into three main fractions: humic acids (HA), which are soluble in alkali and insoluble in acid; fulvic acids (FA), which are soluble in alkali and acid; and humins, which are insoluble in both alkali and acid. Humic substances comprise an extraordinarily complex, amorphous mixture of highly heterogeneous, chemically reactive yet refractory molecules (MacCarthy 2001). Most data on humic substances refer to average properties and structures of a large ensemble of diverse molecules. The precise properties and structure of a given humic extract depends on the particular substrate chosen and the specific conditions of extraction. Nevertheless, the average properties of all HA, FA and humins are remarkably uniform (Stevenson 1994). Humic substances are comprised mainly of aromatic, aliphatic, phenolic, quinonic and N-derived components, which are covalently bound through C-C, C-O-C and N-C bonds. They have an abundance of oxygen-containing functional groups (carboxyl, phenol, alcoholic ketone, ester, and ether) which dominate their properties and structure. Elemental analyses of HA, FA, and humins from all over the world are essentially consistent. Their contents usually range from ~40 to 50 % C and ~40 to 50 % O for FA; ~50 to 65 % C and ~30 to 40 % O for HA. The H, N and S contents range from ~3 to 7 %, ~0.8 to 4.3 % and ~0.1 to 3.6 % respectively, in all of the fractions (Stevenson 1994).

The average molecular weights (MW) of humic substances, HA and, in particular, humin, are still under debate. A small minority of researchers state that HAs are relatively small humic molecules that only self-assemble into apparently high-MW material held together by weak dispersive forces, such as van der Waals, π-π and CH-π interactions (Piccolo 2001; Sutton & Sposito 2005). Most of the research community however claims that HAs are comprised of macromolecules ranging mainly from about 5,000 to 100,000 Da, and less than 10,000 Da for FA (e.g. De Nobili & Chen 1999; Stevenson 1994). To support the latter hypothesis, lignin, a major source for HS, can be taken as an example. Lignin is a high-MW macromolecule. It is degraded by microorganisms (mainly fungi) through oxidative reactions. The high-MW transformation products of lignin degradation are likely to be macromolecules.

A proposed novel structure for soil HA is presented in Fig 1. This structure is based on a large set of average data of published structural information as follows (Stevenson 1994): MW- 6386; Elemental analysis (%): C- 53.9; N- 5.0-; H- 5.8; O- 35.1; S- 0.5; C/N: 10.7; Functional groups (cmol/g): carboxyl- 376; phenol – 188; total acidity – 564. The distribution of % C based on NMR analyses is: aliphatic – 18.1, aromatic – 20.9, carbohydrates 23.7, methoxy – 4.9, carboxylic – 8.4, keton – 4.5, phenolic – 4.2, other groups – 15.3 (Stevenson 1994). To date, published schematic models have overlooked this information even though it is widely accepted in the organic matter literature.

The 3D structure of humic substances is influenced by their basic structure, as well as by chemical and physical properties of the environment such as acidity, ionic strength and humidity. 3D structure also heavily influences their bioavailability and hence their biodegradability.

During the past three decades, our understanding of humic substances has been advanced by development of techniques such as pyrolysis gas chromatography-mass spectrometry (GC-MS), thermochemolysis with tetramethylammonium hydroxide (TMAH) coupled to GC-MS, modern soft-ionization MS, solid-state nuclear magnetic resonance (NMR) spectroscopy based on ^{1}H, ^{13}C, ^{15}N and ^{31}P nuclei measured under various configurations (Hatcher et al. 2001), different size-exclusion chromatography methods, fluorescence spectroscopy and Fourier-transform ion cyclotron resonance MS (ICR-FTMS) (Hertkorn et al. 2006). Biodegradation studies have generally focused on each of the fractions (HA, FA) separately, or on using labeled synthetic materials such as ^{14}C macromolecules made from catechol (Steffen et al. 2002). The latter are far from being representative of natural HA. These methodologies have contributed to the mechanistic understanding of the process. However, in the environment, humic substances cannot be divided into artificial fractions such as HA, FA and humin: they are comprised of a mixture of associated OM and usually interact with other compounds, such as oxide and hydroxide minerals. Furthermore, in nature, some fractions may not be available for biodegradation because of physical barriers and their interactions with other compounds in the environment.

3. The significance of fungi in humus turnover

Microorganisms are the driving force behind the formation, transformation, degradation and mineralization of humic substances. Although bacteria dominate the environment and participate in turnover of humic substances, their ability

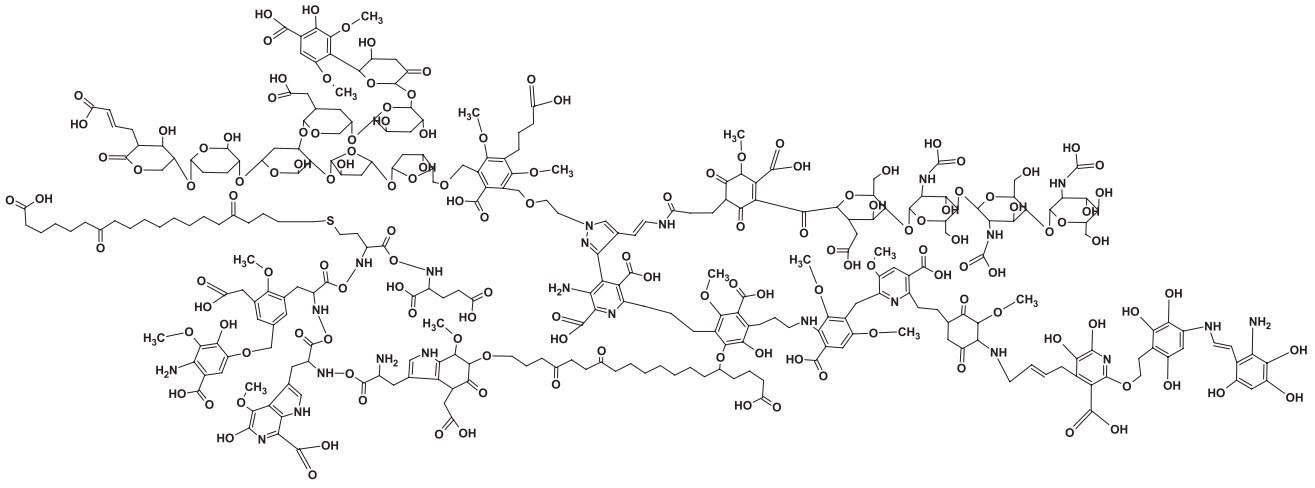

Fig. 1 – Proposed macromolecular structure of a soil humic acid (HA) based on the following common characteristics: MW- 6386 Da; Elemental analysis (%): C- 53.9; N- 5.0-; H- 5.8; O- 35.1; S- 0.5; C/N: 10.7; Functional groups (cmol/g): carboxyl – 376; phenol – 188; total acidity – 564; Distribution of % C based on NMR analyses: aliphatic – 18.1, aromatic – 20.9, carbohydrates 23.7, methoxy – 4.9, carboxylic – 8.4, keton – 4.5, phenolic – 4.2, other groups – 15.3 (Stevenson 1994). The structure was built using the ACD/ChemSketch program.

to degrade stable macromolecules such as HA and lignin is limited (Dehorter *et al.* 1992; Esham *et al.* 2000; Filip & Tesarova 2004; Gramss *et al.* 1999; Machnikowska *et al.* 2002; Tuomela *et al.* 2000). Bacteria probably have their effect *via* the utilization of low-MW compounds such as FA and HA metabolites. Fungi, on the other hand, are the most efficient HS degraders. A recent study describing a biofilm consortium that was enriched by using HA as the sole carbon and energy source suggests that biodegradation is carried out mainly *via* secondary or cometabolic processes, probably by fungi (Qi *et al.* 2004).

In natural ecosystems, saprotrophic fungi act as primary, secondary and tertiary decomposers which feed on and recycle large amounts of carbon as well as other nutrients. They affect plant succession and soil stabilization and are therefore central to terrestrial environment function. Nevertheless, there is still a gap in our knowledge of their actual diversity and function in OM decomposition. Fungi active in the decomposition process include mainly ascomycetes and basidiomycetes which are common in the upper layer of forest and grassland soils. However, their relative abundance and role during turnover of humic substances are still unclear (Deacon *et al.* 2006; O'Brien *et al.* 2005). Approximately 8,500 described species of basidiomycetes are lignocellulose-degrading saprotrophs, and about half of these occur in soil and on fallen plant litter (Lynch & Thorn 2006). Until recently, basidiomycetes were considered less common in habitats such as agricultural soils. However, soil DNA amplification has revealed much greater diversity than was anticipated in this habitat on the basis of culture-based methods or surveys of fruiting bodies (Lynch & Thorn 2006). A recent study (Carney *et al.* 2007) linked to global warming revealed that over a 6 y period under elevated CO_2, soil carbon was reduced by half, with this decline driven by the activity of the soil microbial community. Soils exposed to elevated CO_2 had higher relative abundances of fungi and higher activities of soil carbon degrading enzymes, emphasizing the role of fungi in humic substance turnover.

4. Degradation and transformation of HS by basidiomycetes

Due to their large size, HA macromolecules are not likely to be taken up by microbial cells; they are therefore initially degraded by extracellular enzymes (Kastner & Hofrichter 2001). Many basidiomycetes belong to the white-rot fungi (WRF) and litter-decomposing fungi (LDF). These are considered the most efficient lignin degraders thanks to their nonspecific oxidizing enzymes: manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase (Hatakka 1994; Kirk & Farrell 1987). The recently completed genome sequence of *Phanerochaete chrysosporium* also suggests that many other extracellular enzymes are involved during lignin degradation (Kersten & Cullen 2007). These enzymes (making up the so-called ligninolytic system) lead to the formation of unstable compounds (e.g. phenoxy and carboxy radicals), which can then undergo either condensation and polymerization (humification) (Catcheside & Ralph 1999; Chefetz *et al.* 1998; Zavarzina *et al.* 2004) or further degradation, and even mineralization (Hofrichter *et al.* 1999; Steffen *et al.* 2002). The pathway followed by each enzymatic product (degradation or polymerization) is probably dependent not only on the enzymes and substrates involved, but also on reaction conditions, such as pH, humidity, percent oxygen and electrical conductivity, as well as on the presence of other compounds. Moreover, the enzymatic systems differ from one fungus to the next and are highly dependent on a broader arsenal of enzymes which support the ability to degrade recalcitrant macromolecules. Due to the unique ability of nonspecific oxidizing enzymes to react with a variety of

aromatic substrates, white rot fungi have been found to be the most efficient degraders of humic substances (Hofrichter & Fritsche 1996; Gramss et al. 1999).

Burges & Latter (1960) were the first to show that white rot fungi are able to bleach and degrade HA originating from podzolic soils. Furthermore, they noted that culture aeration is correlated to decomposition level (Burges & Latter 1960; Hurst et al. 1962). This association has been found in many other studies of both lignin and HA degradation. Bleaching of humic substances has been considered an indication of degradation rate, with a dark color (measured at 400–600 nm) representing a higher concentration of humic substances (Stevenson 1994). Cohen and Gabriele (1982) showed that *Trametes versicolor* and *Poria monticola* (brown-rot fungi) were able to degrade and solubilize lignite coal. Infrared analysis revealed differences between the original lignite and the soluble products. In continuing studies, they identified the coal-solubilizing agent as ammonium oxalate (Cohen et al. 1990; Wilson et al. 1987). Other biosolubilization mechanisms of coal by fungi have been found to involve the generation of alkaline metabolic products followed by ionization of the acidic groups (Quigley et al. 1989), or the generation of chelators able to sequester polyvalent ions from the coal, leading to solubilization.

The motivation for studying the depolymerization of coal was to elucidate the coal's molecular structure and to identify novel chemical feedstocks. Ralph and Catcheside were one of the main groups to study this phenomenon. They used the white rot fungus *Phanerochaete chrysosporium* and humic substances extracted from Morwell brown coal by various methods using alkali solutions. Some of the fractions were methylated (using $(CH_3)SO_4$ and CH_2N_2) or demethylated (according to Olson & Diehl 1985). *P. chrysosporium* was found to convert about 85 % of the solubilized Morwell brown coal to lower MW fractions after 16 d of incubation. *In-vivo* and *in-vitro* studies revealed that LiP, H_2O_2 and veratryl alcohol (VA) are crucial to this process, suggesting that the degradation was mediated by VA^+ rather than direct oxidation. Moreover, only humic substances that were premethylated were depolymerized in this system. The authors suggested that methylation of the phenolic and carboxylic groups in this fraction facilitated their depolymerization by LiP, indicating the importance of the humic substance properties on the biodegradation process (Ralph & Catcheside 1994a; Ralph & Catcheside 1994b; Ralph et al. 1996; Ralph & Catcheside 1997; Ralph & Catcheside 1998a; Ralph & Catcheside 1998b; Ralph & Catcheside 1999). Several other white rot fungi, including *Nematoloma frowardii b19*, *Clitocybula dusenii b11*, *Auricularia* sp. and the basidiomycete strains RBS 1k and RBS 1b, were also studied for their ability to degrade coal HS (Hofrichter & Fritsche 1996; Hofrichter & Fritsche 1997a; Hofrichter & Fritsche 1997b; Hofrichter et al. 1997; Catcheside & Ralph 1999). These fungi were able to degrade HA *via* nonspecific oxidizing enzymes, suggesting that MnP is the most important factor during this degradation. Willmann and Fakoussa (1997) found that the two basidiomycete strains, RBS 1k and RBS 1b, were able to solubilize lignite (brown coal), and that different coal substances could affect the production of their nonspecific oxidizing enzymes. The production of extracellular peroxidases by strain RBS 1k was induced by the addition to culture media of native lignite powder or by different lignite fractions (including humic substances extracted using ethanol and toluol (1:1)-so-called bitumen). On the other hand, the extracellular laccase activity found in strain RBS 1b was stimulated by the addition of water-soluble HA, but inhibited by the addition of bitumen or native lignite powder. Recently, Kabe et al. (2005) studied the degradation of lignite HA by three strains of white rot fungi isolated from woods in Japan and found a positive correlation between high MnP activity and HA degradation.

Haider and Martin (1988) were the first to detect direct mineralization of natural HA by white rot fungi. *P. chrysosporium* mineralized up to 56 % of ^{14}C-labeled HA (originating from ^{14}C-wheat straw) and ^{14}C-xenobiotics bound to HA. Wunderwald et al. (2000) showed that halogenated HA, resulting from the humification of xenobiotic compounds (bound residues), can be eliminated by ligninolytic fungi such *N. frowardii* and their MnP system (Wunderwald et al. 2000).

An ecological study by Gramss et al. (1999) examined the ability of 36 fungal isolates (divided according to their ecophysiological functional group—8 white rot fungi, 2 brown-rot fungi, 7 terricolous basidiomycetes, 10 ectomycorrhizal fungi and 9 soilborne and plant pathogens) and 9 bacterial isolates to degrade humic extracts from forest soil (similar to HA—extracted using NaOH). They showed that the so-called wood-degrading fungi are the most efficient HA degraders. However, no significant correlations between the activities of the extracellular oxidoreductases and HA degradation were observed. Nevertheless, purified commercial enzymes such laccase and glucose oxidase caused slight decolorization, while horseradish peroxidase (HRP) caused remarkable bleaching (−18.3 %) of the HA. Catalase and tyrosinase did not bleach the HA.

Another ecological overview was performed by Steffen et al. (2002), who screened 37 fungi (LDF and WRF) from Finnish forests and grasslands and an additional 15 strains from culture collections. Thirty-four of them were able to bleach forest-soil HA on agar plates. The authors focused on the litter decomposing fungus *Collybia dryophila* and found that it could degrade forest-soil HA and mineralize synthetic ''^{14}C-HA'' synthesized from catechol. Moreover, under liquid conditions, the degradation was highly dependent on MnP levels and supplementation of Mn^{2+} ions (Steffen et al. 2002). Recently we isolated and identified two white rot fungi (*Phanerochaete* sp. Y6 and *Trametes* sp. M23) from biosolid compost. These isolates, when compared to the model white rot fungi *T. versicolor* and *P. chrysosporium*, showed the ability to degrade HA extracted from the biosolid compost. Interestingly, only *Trametes* sp. M23 bleached leonardite HA, which is considered to be a highly aromatic and stable type of natural OM (Granit et al. 2007).

Biodegradation of different humic substances by different white rot and litter decomposing fungi revealed different patterns in the production of nonspecific oxidizing enzymes involved in the process. For example, *T. versicolor* was found to secrete laccase as a major extracellular enzyme while *P. chrysosporium* (which has no laccase) secretes peroxidases as major enzymes. These two strains bleached lignite HA to the same extent (Blondeau 1989; Dehorter and Blondeau 1992; Fakoussa & Frost 1999; Temp et al. 1999). Furthermore, different nitrogen and carbon sources, C/N ratios, humic

substance concentrations and properties, and the addition of microelements and mediators all affect this process. The different conditions influence both fungal growth and secretion of nonspecific oxidizing enzymes, thereby affecting the biodegradation process (Catcheside & Ralph 1999; Fakoussa & Frost 1999). Summary information on Basidiomycetes (WRF and LDF) and Ascomycetes, their humic substance substrates, bleaching levels (%) and main enzymes detected during bleaching is presented in Table 1.

5. The role of the nonspecific oxidizing enzymes in HS transformation

Most of the basic research into extracellular nonspecific oxidizing enzymes in white rot fungi has focused on their role in lignin degradation (Higuchi 2004; Kirk & Farrell 1987). However, it appears that they also play an important role in various OM-rich environments in the formation, transformation

Table 1 – Degradation of humic substances (HS) by basidiomycetes (both white rot and litter decomposing species) and ascomycetes

Fungi	Type of HS	Bleaching (%)	Major enzymes detected	Reference
Basidiomycetes (WRF,LDF)				
Trametes versicolor	Forest soil HA	~80	MnP and LiP	Dehorter and Blondeau 1992
	Lignite HA	~80	Laccase	Fakoussa & Frost 1999
Coriolus consors	Umbric Adosol 1 (soil HA)	25.9	ND	Yanagi *et al.* 2002
	Umbric Adosol 2	27.7		
	Dystric Cambisol 1 (soil HA)	41.6		
	Dystric Cambisol 2	51.1		
	Fibric Histosol (soil HA)	29.3		
	HA from 15 different soil samples	~18–40	ND	Yanagi *et al.* 2003
Coriolus hirsusutus	Umbric Adosol 1 (soil HA)	2.6	ND	Yanagi *et al.* 2002
	Umbric Adosol 2	31.1		
	Dystric Cambisol 1 (soil HA)	37.3		
	Dystric Cambisol 2	45.5		
	Fibric Histosol (soil HA)	9.9		
Phanerochaete chrysosporium	Forest soil HA	~75	MnP and LiP	Dehorter and Blondeau 1992
	Lignite HA	~80	Peroxidase	Fakoussa & Frost 1999
	Morwell brown coal	~85	MnP and LiP	Ralph and Catcheside 1994a
Lenzites betulina	Umbric Adosol 1 (soil HA)	0	ND	Yanagi *et al.* 2002
	Umbric Adosol 2	17.5		
	Dystric Cambisol 1 (soil HA)	11.8		
	Dystric Cambisol 2	50.6		
	Fibric Histosol (soil HA)	2.6		
Pleurotus ostreatus	Lignite HA	41.3	MnP and laccase	Gramss *et al.* 1999
	Lignite HA	~50	Laccase	Fakoussa & Frost 1999
Bjerkandera adusa	Forest soil HA	55.5	MnP	Gramss *et al.* 1999
Bjerkandera adusa 59	Brown coal extracted with HCl	~90	laccase	Belcarz *et al.* 2005
	Lessive soil HA	~60	Lipase, Endo-β-1,4-mannanase	
	Chernozem HA	~40	Lipase, Endo-β-1,4-mannanase	
Nematoloma frowardii	synthetic ''HA'' derived from 3-fluorocatechol	~45–60	MnP and laccase	Wunderwald *et al.* 2000
	Coal derived HA	~80	MnP	Hofrichter and Fritsche 1997b
Clitocybula dusenii	Forest soil HA	56.2	MnP and laccase	Gramss *et al.* 1999
Peacilomyces farinosus	Lignite HA	~45	Laccase	Fakoussa & Frost 1999
Collybia dryophila	soil litter HA	~60–80	MnP and laccase	Steffen *et al.* 2002
Gymnopilus sapineus	soil litter HA	45.9	Laccase	Gramss *et al.* 1999
Hypholoma fasciculare	soil litter HA	53.8	MnP and laccase	Gramss *et al.* 1999
Kuehneromyces mutabilis	soil litter HA	41.9	MnP and laccase	Gramss *et al.* 1999
H. frowardii	soil litter HA	73.1	MnP and laccase	Gramss *et al.* 1999
Stropharia rugoso-annulata	soil litter HA	56.7	MnP and laccase	Gramss *et al.* 1999
Pycnoporus cinnabarinus	Lignite HA	~70	Laccase	Temp *et al.* 1999
Polyporus ciliatus	Lignite HA	~40	Laccase and MnP	Temp *et al.* 1999
Ascomycetes				
Paecilomyces inflatus	Compost HA	~30	Laccase	Kluczek-Turpeinen *et al.* 2005
Alternaria Alternaria	Soil HA	~15	MnP	Rezacova *et al.* 2006
Phoma sp.	Soil HA	~18	MnP	Rezacova *et al.* 2006
Clonostachys rosea	Soil HA	~27	MnP	Rezacova *et al.* 2006
Paecilomyces lilacinus	Soil HA	~22	MnP	Rezacova *et al.* 2006

and degradation of humus. In addition, these enzymes are being intensively studied in relation to their ability to degrade a large variety of aromatic organic pollutants (Jarosz-Wilkolazka *et al.* 2002; Ikehata *et al.* 2004). Through evolution, different white rot and litter decomposing fungi developed different combinations and properties of these enzymes, and they therefore differ in their abilities and mechanisms for the degradation of recalcitrant compounds such as HA. The biochemistry of these enzymes as well as the physiology and genetics of their production have been intensively reviewed elsewhere (Higuchi 2004; Hofrichter 2002; Kersten & Cullen 2007; Kirk & Farrell 1987).

Fungal laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are a major component of the ligninolytic system of many white rot fungi (Leonowicz *et al.* (2001); thus, although humic substances are much more complex and amorphous than lignin, there is evidence for the role of laccases in their transformation. The occurrence and properties of fungal laccase have recently been reviewed in detail (Baldrian 2006) suggesting that many of the questions related to its role in the environment remain open. Fungal laccases have different optimum pH values for their activity, ranging from 2 to 8. Moreover, different substrates have different optimum pHs, with that of phenolic compounds being at around 7 (Baldrian 2006). These facts may affect the biodegradability of humic substances in nature since their charge and 3D structure are also dramatically affected by pH. White rot and litter decomposing fungi secrete a number of isoenzymes with different properties; for example, *Trametes gallica* has been shown to secrete up to 20 different isoenzymes of laccase (Dong *et al.* 2005) and 17 nonallelic laccase genes were found in the genome of *Coprinopsis cinerea* (Kilaru *et al.* 2006).

These properties may account for the ability of laccases to oxidize a variety of substrates. For example,Temp *et al.* (1999) found that *Pycnoporus cinnabarinus* (which secretes only laccase as extracellular phenol oxidase) can depolymerize coal HA. Zavarzina *et al.* (2004) found that purified laccase from the white rot fungus *Panus tigrinus* can transform different HA in different ways: HA originating from chernozem soil was depolymerized, peat HA was polymerized, and a decrease in the high-MW fraction and increase in the low-MW fraction of HA originating from soddy-podzolic soil was observed. Moreover, they found that all HAs were competitive inhibitors of laccase, and inhibition therefore increased with increasing HA concentration. Based on the properties of the HA, they suggested that hydrophobicity had the strongest effect on inhibition. Keum and Li (2004) studied the inhibitory effect of HA on laccase from *T. versicolor*. They observed strong inhibition by HA (concentration of 150 μM) during degradation of polychlorobiphenyls (PCBs). However, enzyme activity was restored by the addition of 500 μM Cu^{2+}, suggesting that the initial inhibition was due to depletion of Cu^{2+} from the active site of the enzyme (Keum & Li 2004). It should be noted that results obtained *in vitro* may not reflect the situation with whole fungal cultures in natural environments. Evidence of this was reported by Willmann and Fakoussa (1997), who observed that extracellular laccase production could be stimulated by the addition of water-soluble HA, but is inhibited by the addition of other coal fractions or by native lignite powder.

Lignin peroxidase (EC 1.11.1.). Strong evidence for the involvement of LiP in degradation of humic substances was found during the incubation of *P. chrysosporium* with brown coal using two different media, one which suppressed LiP and MnP (high N) and one which induced both of these enzymes (low N). No decrease in absorbance (400 nm) was found using the high-N medium, while a strong decrease occurred using the low-N medium (Ralph & Catcheside 1999). The crude extract which exhibited high LiP activity was found to depolymerize humic substances (Catcheside & Ralph 1999). In addition, polymerization of humic substances from coal was detected by using a mutant that was unable to produce LiP but synthesized MnP, suggesting that LiP is the key enzyme in degradation. A mutant that does not secrete either of the enzymes failed to depolymerize brown coal (Catcheside & Ralph 1999).

Manganese peroxidase (EC 1.11.1.13) from *N. ftowardii, C. dusenii* and *Collybia dryophila* was found to degrade and mineralize HS originating from brown coal. Moreover, MnP has been suggested as a key enzyme in the degradation and mineralization of HAs (Hofrichter & Fritsche 1997b; Hofrichter *et al.* 1998; Hofrichter *et al.* 1999; Kabe *et al.* 2005; Steffen *et al.* 2002). Ralph and Catcheside (1998b) found that solubilized macromolecules from Morwell brown coal were depolymerized by MnP when incubated under hyperbaric O_2. However, under N_2 or air, they were polymerized, suggesting that a net depolymerization by Mn^{3+} requires molecular oxygen to inhibit the coupling of coal radicals. In contrast, they found that solubilized brown coal inhibited the activity of both MnP and LiP (Ralph & Catcheside 1994a). Mn^{3+} acetate alone was also shown to bleach HA extracted from forest soil (Gramss *et al.* 1999). Hofrichter *et al.* 1999 detected that synthetic ^{14}C labeled HA mineralization by MnP was considerably enhanced in the presence of the thiol mediator glutathione.

Apart from these three enzyme families, other enzymes are known to be involved in lignin degradation, including glyoxal oxidase (GLX), cellobiose dehydrogenase (CDH),aryl alcohol oxidase (AAO),versatile peroxidase (VP) and cytochrome P450 and may also be involved in turnover of humic substances. Recently, a new oxidizing enzyme, peroxygenase, isolated from *Agrocybe aegerita* has been described (Hofrichter & Ullrich 2006). In addition results from the genome analyses of *P. chrysosporium* identified more than 350 possible extracellular genes (out of ~10000 genes) which might participate in lignin and humic substance degradation. A proposed scheme for the degradation and transformation of humic substances by white rot and litter decomposing fungi is presented in Fig 2.

6. Chemical and physical changes in humic substances during incubation with white rot and litter decomposing fungi

Relative to enzymatic aspects, there is significantly less information regarding chemical and physical changes undergone by humic substances during biodegradation. Their structural complexity makes the analytical detection of changes extremely difficult. Not only is it hard to characterize humic substances, the secretion of enzymes and other compounds and

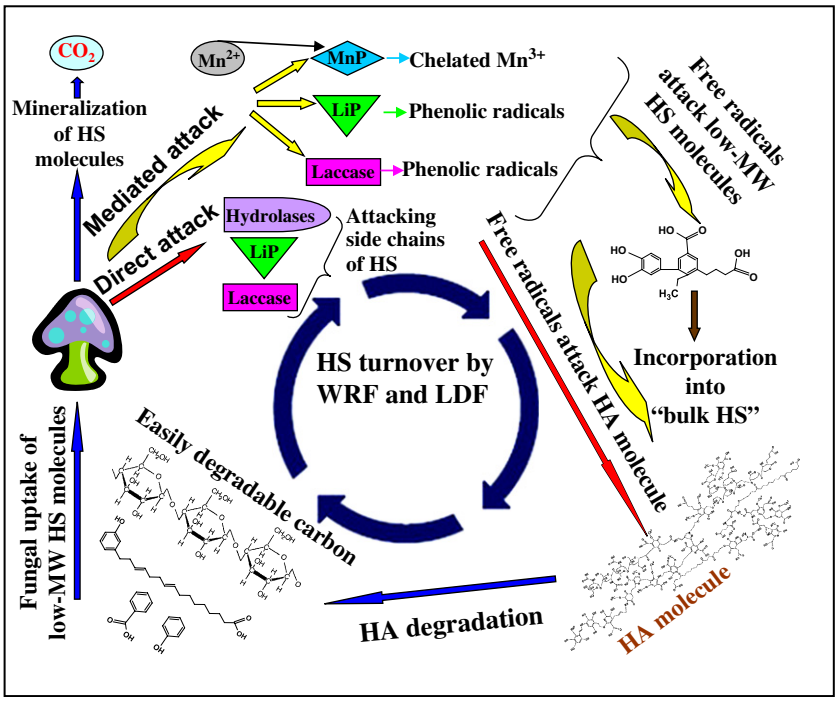

Fig. 2 – Proposed model for humic substances (HS) degradation and transformation by WRF and LDF. The fungi can attack the HS throw three main pathways: (i) direct attack of HS side chains using hydrolases, laccases and LiP; (ii) mediated attack of low-MW HS which can lead to degradation and mineralization or incorporation and polymerizations; (iii) mediated attack of high-MW HS. To start the cycle, easily degradable carbon sources are required.

the possible sorption of humic substances to the mycelium are liable to cause difficulties in interpretation. Different size-exclusion chromatography methods are commonly used to show that bleaching of humic substances is associated with degradation (Blondeau 1989; Gramss *et al.* 1999; Hofrichter *et al.* 1998; Ziegenhagen & Hofrichter 1998). Nevertheless, the accuracy of humic substances MW and size determinations has been criticized due to changes that occur with different methods and conditions (De Nobili & Chen 1999). HA mineralization has been demonstrated by using ^{14}C-labeled synthetic ''HA'' (Hofrichter *et al.* 1999; Steffen *et al.* 2002). Unfortunately, this ''HA'' is far from being representative of natural ones. Using a much more representative method, Haider and Martin (1988) detected mineralization of HA originating from ^{14}C-wheat straw. Dehorter *et al.* (1992) incubated both *P. chrysosporium* and *T. versicolor* with HA originating from forest soil and did not notice any change in HA properties (detected using ^{13}C-NMR) after 30 % loss of HA. However, in a contradictory study, incubation of the white rot fungal strain RBS 1k with HA originating from lignite resulted in an increase in carboxylic groups and hydroxylated and methoxylated aliphatic groups, together with a decrease in aromatics. Elemental analysis showed an increase and decrease in O and H content, respectively (Willmann & Fakoussa 1997). Similar results were observed during solubilization of Rhenish brown coal by *Lentinula edodes* and *T. versicolor* (Gotz & Fakoussa 1999). Ralph and Catcheside (1999) found, by GC-MS analysis, that semi-purified LiP from *P. chrysosporium* can depolymerize coal humic substances to low-MW fragments containing between 9 and 12 C atoms. All the fragments except one contained O. Yanagi *et al.* (2002, 2003) studied the influence of different HA properties on biodegradation and found that the H/C and O/C ratios (taken from the elemental analysis) and aromaticity (measured by ^{13}C-NMR) are strongly correlated with the degree of soil-HA degradation by *Coriolus consors*. Our recent results regarding 3 different HAs and 4 different white rot fungi support these findings (Granit *et al.* 2007).

In the last three decades, there has been great progress in the analytical methods which can be used to characterize humic substances. By applying these methods during biodegradation, a better understanding of the mechanisms governing this process can be achieved.

7. Degradation and transformation of HS by ascomycetes

Although ascomycetes are common soil inhabitants, their utilization of humic substances has been studied to a lesser extent than that of white rot and litter decomposing fungi, leaving many open questions. Their ability to degrade stable compounds such as HA is limited compared to white rot and litter decomposing fungi (Fakoussa & Frost 1999; Kluczek-Turpeinen *et al.* 2005; Tuomela *et al.* 2000). Nevertheless, they are common in environments such as forests, grasslands, soils and compost and contribute to the turnover of humic substances (Kluczek-Turpeinen *et al.* 2005; Rezacova *et al.* 2006).

In a similar fashion to basidiomycete species, ascomycetes such as *Penicillium*, *Fusarium*, *Trichoderma* and *Aspergillus* species have also been investigated for their ability to produce clean fuels and chemical feedstocks from coal (Achi 1994; Catcheside & Ralph 1999; Fakoussa and Hofrichter 1999; Holker *et al.* 1995; Laborda *et al.* 1999; Yuan *et al.* 2006). Two ascomycetes, *Fusarium oxysporum* and *Trichoderma atroviride*, were found to solubilize coal *via* the synergistic effects of a number of cellular mechanisms. *F. oxysporum* appears to solubilize coal by increasing pH and by the action of chelators induced during growth (without the involvement of enzymes). *T. atroviride* appears to use, in addition to an alkaline pH and high chelator activity, at least two classes of enzymatic activity to attack coal: hydrolytic activity for coal solubilization and ligninolytic activity for HA degradation (Holker *et al.* 1999).

Numerous studies have focused on the role of ascomycetes in humus formation and modification. *Epicorom nigrum*, *Eurotium echinulatum*, *Hendersonula torulidea* and *Aspergillus sydowi* were found to synthesize HA from different phenolic materials under different conditions. Polymerization was associated with phenoloxidase enzymes. Addition of different N sources as well as clay minerals enhanced growth, phenol synthesis and phenolic polymer formation (Martin *et al.* 1967; Martin *et al.* 1972; Bondiett *et al.* 1971; Haider & Martin 1967; Martin & Haider 1969). During composting, humification occurs at an accelerated rate compared to that in soil; the intermediates and final products formed during composting resemble those of OM decomposed in natural aerobic environments such as litter layers and soil-surface horizons (Inbar *et al.* 1990). Thus, composting may be useful as an infrastructure for research into humic substance transformation. Chefetz *et al.* (1998) isolated the laccase-producing fungus *Chaetomium thermophilium* from municipal solid-waste compost during its thermophilic stage. Incubation of this laccase with the humic fraction of the water-soluble OM obtained from municipal solid-waste compost and guaiacol resulted in polymerization. The authors suggested that this enzyme is involved in the humification process during composting. Kluczek-Turpeinen *et al.* (2005) found that two strains of *Paecilomyces inflatus* isolated from compost are able to mineralize 5 % of ^{14}C-labeled synthetic HA (made from ^{14}C-labeled catechol). In addition, they detected up to 30 % decolorization of natural compost HA and a moderate change in the MW distribution of both the HA and FA fractions. Laccase activity was enhanced in media containing HA, indicating involvement in HA modifications.

Recently, several commom microfungal species (species of *Alternaria*, *Clonostachys*, *Exophiala*, *Penicillium*, *Fusarium*, *Phoma* and *Paecilomyces*) have been studied for their ability to modify soil HA and FA. These fungi slightly decolorized HA (up to 27 %) and therefore increased the E_4/E_6 ratio (indicating a decrease in HA MW; Chen *et al.*, 1977). Much smaller changes were observed during the incubation of FA (Rezacova *et al.* 2006). These results are in agreement with Gramss *et al.* (1999) who showed that HAs were more easily degraded than FA. Unlike humic substance degradation by white rot and litter decomposing fungi, which is considered to be a cometabolic process, no significant effect of glucose on the utilization of either HA or FA was observed (Rezacova *et al.* 2006).

8. Conclusions

Although the formation, transformation and degradation of humic substances by fungi in the environment are important processes, their research is only in its infancy. In a similar fashion to lignin, degradation and transformation of refractory humic substances by white rot and litter decomposing fungi occur only in the presence of other easily degradable C sources. The details of the process are far from being understood, differ from one fungus to another, change in diverse environments and depend on the substrate. Questions concerning the byproducts of the process, the role of each of the non specific oxidizing enzymes, the differences between fungal species and the effects of the properties of humic substances on biodegradation still remain open. Moreover it seems that hydrolases and other not yet discovered enzymes are likely to be involved. Nevertheless, a basic pattern can be discerned: oxidation of humic substances and formation of unstable radicals by nonspecific oxidizing enzymes occurs, which then leads to two distinct potential pathways: (i) degradation and some mineralization of humic substances; or (ii) transformation and polymerization of humic substances. The conditions leading to each of these contrasting pathways remain unclear. However, aeration, nutrients, and probably pH and moisture are strongly associated with it. Due to the complexity of the process and its chaotic nature, numerous specific pathways probably exist. Thus, there seems to be much more to this process than initially meets the eye.

The major questions which are still under debate are the precise role of saprophytic fungi in humus degradation and turnover, and the impact of each taxon. We suggest that fungi play a key role in the formation, transformation and degradation of humic substances, especially in forests and grasslands which contain large amounts of OM and during composting of organic waste. It seems that white rot and litter decomposing fungi are much more active in the degradation and mineralization of humic substances, whereas ascomycetes influence mainly the modification and polymerization of humic materials.

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