

Chapter 10

Heterophase Synthesis of Humic Acids in Soils by Immobilized Phenol Oxidases

A.G. Zavarzina

10.1 Introduction

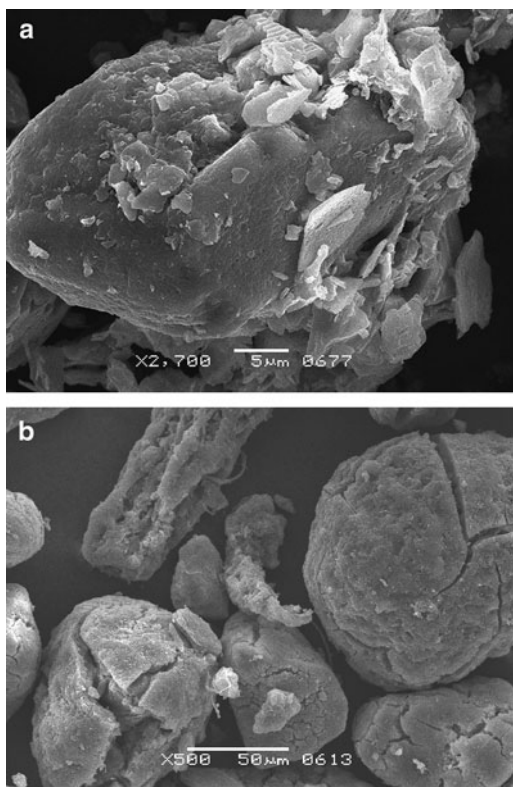
Soil organic matter (humus) is one of the largest carbon reservoirs in the biosphere and holds about 1,500 Pg of C_{org} (Batjes 1996). Humus has a vital significance for the development and functioning of terrestrial ecosystems. Two major processes are responsible for C_{org} accumulation in soils: (1) humification, leading to formation of recalcitrant humic substances (HS); (2) organo-mineral interactions leading to chemical (via adsorption) or physical (occlusion within aggregates) stabilization of organic molecules. As a result of organo-mineral surface interactions organic coatings of varying thickness are formed on the mineral grains (Fig. 10.1 - former 10.5). The most stable C_{org} fraction in soils with mean residence time of $n \times 10^2$ – 10^3 years is represented by adsorption complexes of humic substances with fine mineral particles (Mikutta et al. 2006). Although clay-sized organo-mineral complexes comprise 50–75% of soil organic matter in cold and temperate soils (Christensen 2001), mechanisms of their formation are not fully understood yet. The concept of sorptive preservation implies that organic matter must occur in a dissolved state prior to adsorption (Guggenberger and Kaiser 2003). This is not in contradiction with formation of fulvic acid complexes with minerals. Fulvic acids (FA) are low molecular weight (0.3–2 kDa) water- and acid-soluble humic compounds, capable of downward migration in the soil profile to adsorption sites. A considerable fraction of soil humus is represented by humic acids (HA), which are highly polydisperse (5–100 kDa) and macromolecular by nature (mean average molecular weight is about 50 kDa). Only low molecular weight HA fractions can move as true solutions from the place of synthesis (e.g., litter) to the underlying mineral soil; mobilization of high molecular weight fractions is only possible as colloids. Indeed, the mean average molecular weight of dissolved organic matter in

A.G. Zavarzina

Faculty of Soil Science, Moscow State University, Moscow 119991, Russia

e-mail: zavarzina@mail.ru

Fig. 10.1 SEM images of organic coatings on the mineral grains: (a) A_h horizon of albeluvisol: thin wave-like coatings on silt-sized particles; (b) B_{hf} horizon of Al-Fe humic podzol: thick coatings consisting of amorphous Al oxyhydroxide–humic acid complexes on the non-weathered surface of primary minerals. The cracks are formed upon coating drying



soil solutions is 1.7 kDa (Perdue and Ritchie 2004). One can assume that HA polymers are formed in situ in mineral soil horizons. A possible mechanism is heterophase polymerization of low molecular weight (thus soluble and mobile) precursor material in presence of catalytically active solid phases. In this chapter, the available data supporting the concept of surface HS polymerization are summarized, and evidence for the key role of immobilized phenol oxidases and solid matrix in the catalytic synthesis of HAs is provided.

10.2 Synthesis of Humic Substances from Soluble Precursors

Two main humification pathways co-exist in soils: (1) synthesis of HS from polymeric precursors (lignins, melanins) by their partial oxidative degradation or (2) synthesis of HS from low molecular weight precursors by their oxidative coupling (Stevenson 1994). While the first pathway (lignin–protein theory) is more typical for wood, litter, or poorly drained peaty horizons, the second pathway should be important way of HA formation in mineral soil layers (Table 10.1).

Table 10.1 Principal differences in humification processes in litter and humus horizons of forest soils

Property	Litter	Humus horizon
Actual enzyme activity	High	Moderate/Low
Main starting material	Particulate organic matter (foliage, twigs, wood at different stages of decomposition)	Leached-down soluble organic substances, root exudates and root decomposition products, microbial metabolites
Initial molecular weight of precursor material	High molecular weight	Low molecular weight
Dominant solid phase	Organic	Inorganic
Dominant process	Solid-state fermentation	Heterophase synthesis
Reactions, leading to HS formation	Oxidative transformation	Precipitation or surface polymerization
Product	Humic colloids	Humus–mineral adsorption complexes

Synthesis of HS from soluble compounds occurs by: (1) oxidative coupling of polyphenols with nitrogenous compounds and other soluble precursors (polyphenol theory); (2) sugar–amino acid condensation (Maillard reaction). The polyphenol theory is more popular and postulates that soluble phenolic substrates are oxidized into highly reactive phenoxy radicals and quinones, which then undergo non-enzymatic spontaneous coupling reactions. Dark-colored heterogeneous structures of varying composition and molecular weight are formed as a result of the process. Polymerization occurs via C–C and C–O coupling of phenolic reactants and N–N and C–N coupling of aromatic nitrogenous compounds (Sjöblad and Bollag 1981). It is widely accepted that HS formation is a catalytic process, rather than auto-oxidation; however, the role of enzymes and abiotic catalysts in synthesis of HS is still under the discussion (Bollag et al. 1998).

10.2.1 Enzymatic Catalysis

Peroxidases (EC 1.11.1.7), laccases (EC 1.11.1.14), and tyrosinases (EC 1.14.18.1) are the major enzymes that catalyze polymerization of phenolic compounds via a free radical mechanism. Peroxidases are heme-containing oxidases catalyzing one-electron oxidation of a broad spectrum of phenolic substrates by H_2O_2 with formation of phenoxy radicals and H_2O (Fig 10.2a). Laccase is a multicopper oxidase that performs four one-electron oxidations of the wide range of substituted phenols and aromatic amines by O_2 with formation of semiquinones and quinones; O_2 is reduced to H_2O (Fig 10.2b). Tyrosinases contain a copper pair at the active site and catalyze two concomitant reactions: *o*-hydroxylation of monophenols yielding *o*-diphenols (monophenolase activity); $2e^-$ oxidation of *o*-diphenols to *o*-quinones (diphenolase activity); O_2 is reduced to H_2O in the course of the reaction (Fig 10.2c).

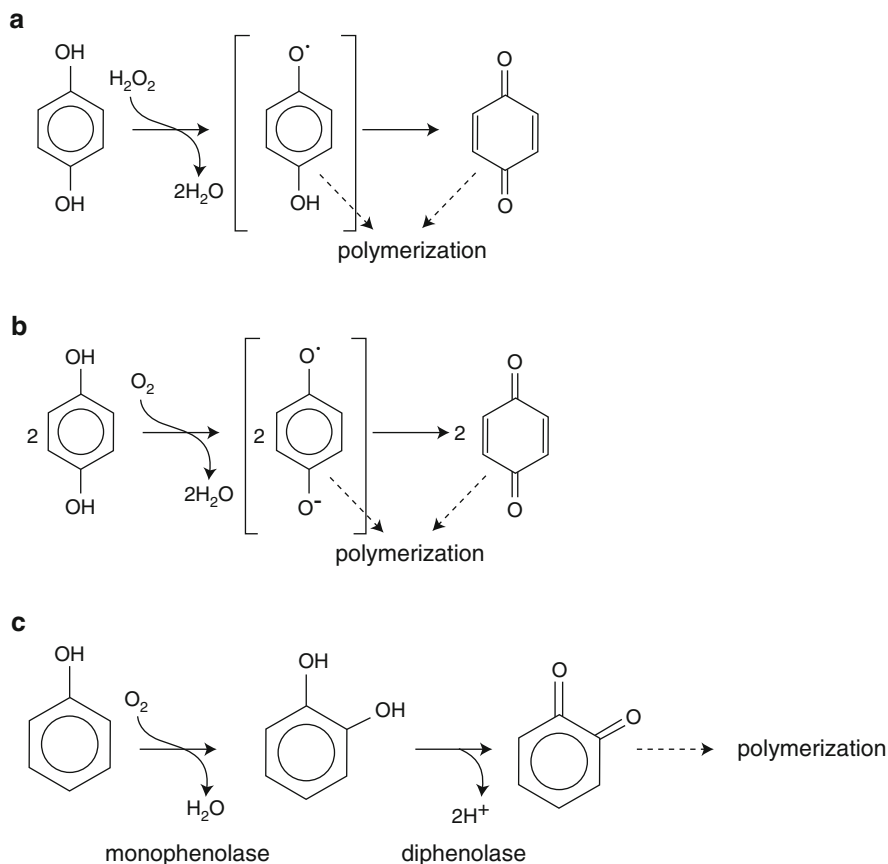


Fig. 10.2 Schematic representation of phenolic substrates oxidation by (a) peroxidase, (b) laccase, and (c) tyrosinase

10.2.1.1 Occurrence of Phenol Oxidases in Soils

Among the enzymes catalyzing humus polymerization, laccases followed by peroxidases are most widespread and common in soils; tyrosinase is less abundant (Criquet et al. 2000; Di Nardo et al. 2004; Snajdr et al. 2008). Fungi are the main source of phenol oxidases in soils (see Chap. 11), although peroxidases and laccases may be also excreted by bacteria and plant routes (Gramss et al. 1998). Phenol oxidase activities in soils exhibit high spatial heterogeneity, more pronounced in litter than in underlying organo-mineral horizons (Snajdr et al. 2008). As a rule, phenol oxidase activities decrease with depth following the decrease in microbial biomass, organic matter content, and its utilizable forms; mineral horizons are characterized by several times lower activities of enzymes than litter (Snajdr et al. 2008). In podzol soils with surface (A_h) and subsurface (B_{hf}) organic-rich horizons two maxima of laccase and peroxidase activities were observed which

correlated with distribution of microfungi, organic matter, and Al (Fe) oxyhydroxides (Zavarzina et al. 2007). Phenol oxidase activities in soils may display optimal moisture levels, above which low oxygenation inhibit activity (Fenner et al. 2005) and below which activity declines due to moisture limitations (Toberman et al. 2008).

10.2.1.2 Phenol Oxidase Distribution among Soil Phases

Soil structure is defined as an arrangement of organic, mineral, and organo-mineral particles, forming aggregates of different size and stability with aqueous phase present in macro-, mezo- and micropores between the aggregates or inside them. Soil enzymes can be distributed among soil aqueous and solid phases upon release from their producers. It is widely accepted that free enzymes are unstable in soil environment and are therefore quantitatively insignificant (Nannipieri and Gianfreda 1998). Immobilized enzymes are more resistant to changes in environmental factors, proteolysis, and inhibitory substances, which allow higher enzyme concentrations to persist in soils (Quiquampoix et al. 2002; Tietjen and Wetzel 2003). Binding of enzymes to solid surfaces is determined by the enzyme isoelectric point, surface area, and charge of solid supports. In mineral soil horizons, most phenol oxidase activity is usually found in the silt and clay-sized fractions (Sarkar et al. 1989; Allison and Jastrow 2006). This fraction contains primary minerals, clay minerals, amorphous metal oxyhydroxides, and humus–mineral complexes, which are <0.05 mm in size. Primary minerals are characterized by low surface area and are therefore poor adsorbents for the enzymes and organic matter. Clay minerals such as smectites and illites as well as humus–clay complexes possess large surface areas ($100\text{--}600\text{ m}^2\text{ g}^{-1}$) but carry an overall negative charge (Tipping and Cooke 1982; Schulze 2002). At pH 4–6, typical for most forest soils, phenol oxidases are also negatively charged (pI 3.0–4.5). This can limit enzyme–mineral interactions due to electrostatic repulsions. Oxyhydroxides of Al and Fe are common and abundant in soils and are present as individual minerals (e.g., gibbsite, goethite) or as coatings on silicates and aluminosilicates. Besides possessing large surface area, Al and Fe oxyhydroxides carry a positive charge at pH < 8.0 (Huang et al. 2002a) enabling strong sorption of negatively charged enzymes. Dominant adsorption mechanisms of phenol oxidases onto the oxyhydroxide surfaces include electrostatic attraction (Ahn et al. 2007) and ligand exchange (Naidja et al. 1997). It was found that pure Al hydroxide adsorbed about nine times more laccase from *Trametes villosa* than other non-crystalline minerals such as ferrihydrite ($\text{Fe}_5\text{HO}_8\cdot 4\text{H}_2\text{O}$) or birnessite ($\delta\text{-MnO}_2$); laccase activity and kinetic properties remained almost unaffected (Ahn et al. 2007). Coating of clay minerals by Al hydroxides favored adsorption of tyrosinase (Naidja et al. 1997). Kaolinite and illite coated by Al hydroxide adsorbed 5–10 times more *Panus tigrinus* laccase than uncoated minerals (Zavarzina 2006a). Thus, oxyhydroxides of Al and Fe can be considered as major inorganic supports for phenol oxidases in soils due to ubiquity, abundance, and surface characteristics, enabling effective enzyme immobilization.

The role of amorphous Al and Fe compounds as surfaces modifiers is particularly important in sandy soils (e.g., podzols), where primary minerals form the bulk of mineral matrix. The correlation between phenol oxidase activities and depth distribution of Al and Fe oxyhydroxides (Zavarzina et al. 2007) additionally supports this contention.

10.2.1.3 Synthesis of Humic Substances in the Aqueous Phase

Polyphenol theory of humus formation is largely based on experiments with homogeneous (monophase) systems where enzymes and their substrates were in dissolved state and reacted with each other in the solution bulk. In the history of the study of HA synthesis, the following work can be mentioned: synthesis of dark-colored humic-like products in mixtures containing pyrogallol, pepton, H_2O_2 , and cell-free culture liquid of *Aspergillus niger* and *Penicillium sp* (Kononova 1966); synthesis of HS in mixtures containing catechin or hydroquinone, amino acids, glucose, and laccase of *Polystictus versicolor* (Trojanowski 1961, as reviewed by Kononova 1966); formation of HS from monophenols, phenolic acids, and N-containing compounds in presence of phenol oxidases (Flaig 1966). Later experiments have shown that condensation in aqueous phase is highly dependent on precursor concentration. At substrate concentrations of 0.5–10 mM, only oligomers (Bollag et al. 1983; Liu et al. 1985; Leontievsky et al. 1999; Zavarzina 2006a), polycondensates with m/z ranges up to 900 (Naidja et al. 1998), or polymers up to 4.0 kDa (Rittstieg et al. 2002) were formed. At high precursor concentrations ($>1 \text{ mg ml}^{-1}$), the molecular weight of the soluble polymer could reach 10 kDa (Zavarzina 2006a); however, further polymerization was terminated by precipitate formation process, which consumed the available monomers. The insoluble product, consisted of high molecular weight fraction ($>75 \text{ kDa}$, minor peak) and low molecular weight co-precipitate (10 kDa, major peak).

Although homogeneous catalysis is important for the understanding of principle reaction mechanisms, it has low relevance to the soils where enzymes are mostly bound to solid surfaces and work in heterogeneous system (see Sect. 10.2.1.1). If it is assumed that polymeric HS are formed in the aqueous phase, the possible mechanism can be so-called *precipitation or adsorption polymerization*. The precipitative polymerization mechanism is well known from organic chemistry, for example, for polyaniline formation (Fedorova and Stejskal 2002, Yagudaeva et al. 2007). The factors that favor this reaction are high monomer concentrations and a chemically inert template with high surface area (e.g., silica gel). If one applies precipitation polymerization to the humus synthesis in soils, the following reactions should occur: (1) phenolic compounds are oxidized at the solid surface by immobilized enzymes to phenoxy radicals and quinones which then (2) dissociate from the enzyme active site and undergo spontaneous coupling in equilibrium solution with (3) subsequent deposition and immobilization of the insoluble (polymeric) product on the solid surface (Fig. 10.3). An example of the laboratory study that apparently mimicked this process was that of Naidja et al. (1997): they

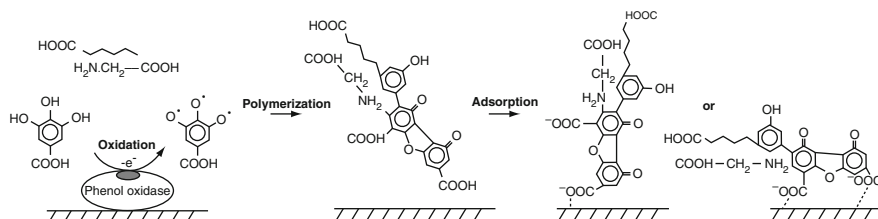


Fig. 10.3 The possible reaction sequence during synthesis of humic substances by precipitation polymerization of monomeric precursors in presence of immobilized laccase

demonstrated that oxidation of dissolved catechol by tyrosinase, immobilized on Al oxyhydroxide-coated montmorillonite resulted in formation of dark-colored products that were adsorbed on the mineral surface and formed organic coating. Infrared spectroscopy revealed similarity of the adsorbed compounds to natural HSs. Precipitation polymerization could also lead to polymeric precipitate formation in some abiotic systems when primary minerals or metal oxides are used as abiotic oxidants (see Sect. 10.2.2). However, in the natural soil environment the formation of polymeric HS on soil minerals by precipitation polymerization is questionable for the following reasons:

1. Monomeric substrate condensation to insoluble products requires high solution concentrations ($>1 \text{ g L}^{-1}$). Average concentrations of dissolved organic carbon in natural environment are several orders of magnitude lower: 0.1 mg L^{-1} in groundwater and up to 100 mg L^{-1} in peat bogs (Klavinš 1997; Perdue and Ritchie 2004). No formation of insoluble polymeric product can be expected at such conditions.
2. If it is assumed that the soil solution can be concentrated to appropriate levels (e.g., upon drying), the presence of charged solid surfaces should interfere in polymerization process in the aqueous phase. Radical self-coupling (coupling with each other) dominates in systems that lack appropriate solid surfaces to participate in cross-coupling (Huang and Weber 2004). Charged solid surfaces, in addition to potentially binding phenoxy radicals, can adsorb original phenolic substrates, reducing their concentration in soil solution. Fast adsorption of dissolved organic matter, and especially of phenolic compounds onto soil mineral phases, is a well-known phenomenon (Lehmann et al. 1986; Dalton et al. 1989; Gallet and Pellissier 1997; Kalbitz et al. 2000). Adsorption is largely irreversible (Lehmann and Cheng 1988; Cecchi et al. 2004), resulting in low concentrations of individual phenolic acids in both the aqueous phase and soil extracts. For example, in soddy-podzolic soils, amounts of ethanol-extractable phenolic acids were $15\text{--}150 \text{ }\mu\text{g}$ per 100 g of soil (Kuvaeva 1980), while average amount of identifiable lignin-derived phenols in soil solutions comprised 0.6% of DOM (Perdue and Ritchie 2004).
3. If it is assumed that temporal increase in concentration of soil solution occurs and the soil mineral phase is inert and does not adsorb enzyme substrates and

monomeric reaction products (e.g., elluvial horizons in podzols, consisting largely of weathered primary minerals), then the polymerization in the solution bulk will be limited by the reaction kinetics. The polymerization process leading to precipitate formation is slow (>24 h) even in homogeneous systems (Kononova 1966; Zavarzina 2006a). In heterogeneous systems (especially in an unstirred medium) the diffusion of substrates to the active site of the enzyme becomes more limiting.

4. And finally, the problem with polymerization in dilute solution lies also in thermodynamics of the polymerization reaction (Lambert 2008). Taking 0.5 M glycine solution as an example, Lambert (2008) has demonstrated that successive polymerization events in solution lie further and further up on the G° scale, making polymer formation in aqueous phase unfavorable.

It can be thus concluded that the synthesis of high molecular weight HAs is barely possible in the aqueous soil phase under natural soil conditions. Formation of only fulvic acid-like products can be expected. Accepting that polymeric HA (50–100 kDa) do exist as coatings on soil minerals, some other mechanisms than homogeneous catalysis or precipitation polymerization should be responsible for their formation if not only they originate from humic colloids that undergo solubilization (Sect. 11.4.2.1) and subsequent adsorption.

10.2.1.4 Synthesis of Humic Substances on the Solution/Solid Interface

As discussed in previous section, the following factors should be kept in mind when dealing with humus formation from soluble precursors in natural soil systems: (1) substrate concentrations in the bulk soil solution are very low; (2) enzymes are present in an immobilized form; (3) monomeric phenolic compounds leached from the forest floor or excreted by plant roots become rapidly and irreversibly adsorbed onto solid soil matrix. It is generally accepted that low extractability of phenolic compounds from soils is a consequence of their high reactivity at solid surfaces resulting in the oxidative cross-coupling to solid phase or polymerization. Thus, it is reasonable to assume that synthesis of polymeric HS from soluble precursors in mineral soil horizons proceeds on the solid–solution interface and not in the solution bulk. The following experimental data support this concept:

1. At low solution concentrations, the presence of interfaces substantially accelerates the rates of substrate coupling in comparison to solid-free systems (e.g., Huang et al. 2002a, b). This effect is explained by the concentration of monomers on surface due to e.g., electrostatic attraction (Danielewicz-Ferchmin and Ferchmin 2004), which helps to overcome the energetic barrier to polymerization, making polymerization thermodynamically favorable (Lambert 2008).
2. Direct experimental evidence exists that polymerization reactions at the surface of a solid support precede polymerization in the supernatant solution, even at high monomer concentrations (Fedorova and Stejskal 2002, Sapurina et al. 2003). Polymerization in adsorbed state (so-called *surface or boundary*

polymerization) gives rise to organic coatings that consists of polymeric, partially non-extractable compounds.

Although surface polymerization process is well known from polymer science (Sapurina et al. 2002; Boufi and Gandini 2002), experimental data that demonstrate its application to humus chemistry are rather scarce. Surface polymerization instead of precipitation polymerization could well have occurred in the study of Naidja et al. (1997) (see Sect. 2.1.3), but unfortunately molecular weight of the mineral-bound reaction product was not measured. To fill this gap, we have made an attempt to demonstrate the possibility of high molecular weight HAs formation by surface polymerization of monomers in the presence of immobilized fungal laccase, and to elucidate the effect of the nature of the mineral support and the role of biotic catalysts in the polymerization process (Zavarzina 2006a, b). It is necessary to outline briefly the experimental design used, to show that the polymerization process proceeded at solid–solution interface. First, purified fungal laccase was immobilized by adsorption on kaolinite, kaolinite–hydroxyaluminum complex, illite, or montmorillonite. After washing of enzyme–mineral complexes with acetate buffer (pH 4.5), precursor mixture solution containing gallic, caffeic, ferulic, hydroxybenzoic, vannilic acids, tryptophan, and tyrosin was added. On the basis of preceding adsorption experiments, the concentration of precursor solution was selected so as to achieve maximal adsorption of monomers and multilayer formation. After 15 min of the reaction period (no polymerization in the bulk of the reaction mixture occurred according to HPLC analysis of supernatants and mono-phase controls), the mixtures were centrifuged, the supernatant solutions with unbound monomers were removed, and the pellets containing enzyme and adsorbed precursors were rapidly washed with acetate buffer. Then, fresh buffer was added to the pellets and the mixtures were incubated at room temperature in the dark for 24 h without agitation. Initially white kaolinite-based supports became brown within 15 min of adsorption stage; during further incubation period, the mineral staining became progressively darker in color. After 24 h, the alkali-extractable products were analyzed by spectroscopic methods and gel filtration. They were found to be polymeric (molecular weights from 5 to >75 kDa), resembling soil HA by visible and infrared spectra and molecular weight distributions (Fig. 10.4). Addition of EDTA to the extracts in order to destroy possible metal bridges between HA “subunits” caused only slight reduction in the amount of high molecular weight fraction, suggesting that the extracted reaction products were true macromolecular by their nature. It should also be emphasized that some portion of high molecular weight products could well have been retained by the clay surfaces as the extraction was not complete. The peak of high molecular weight fraction was largest in the extract from Al oxyhydroxide-coated kaolinite. This is possibly because this mineral adsorbed the largest amount of monomers and had the lowest suppressing effect on the laccase activity compared to the other minerals used. HA-like products formed on montmorillonite were most polydisperse, suggesting that surface morphology of mineral supports may be another important factor that determines molecular weight distribution pattern of the polymeric product. No high molecular

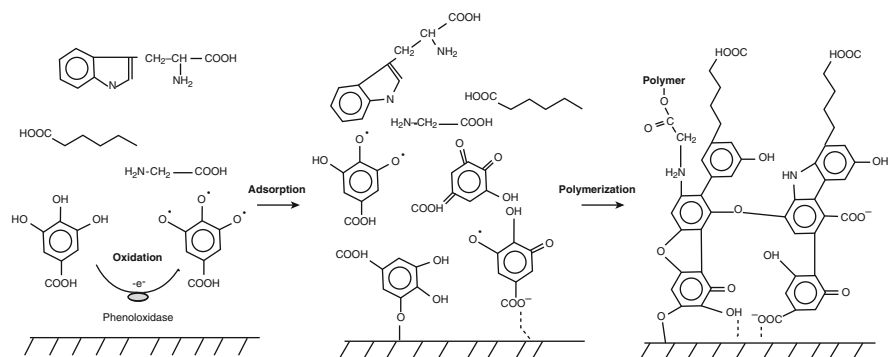


Fig. 10.4 The possible reaction sequence during synthesis of humic substances by surface polymerization of monomeric precursors in presence of immobilized laccase

weight fraction formation was observed in parallel monophasic experiments in which the same enzyme activities and substrate concentrations as those reacted on solid surfaces were used. No products with molecular weight larger than 5 kDa were formed on the minerals in absence of immobilized laccase (abiotic controls). Surprisingly, humic-like polymers were formed on hydroxyaluminum kaolinite even at reverse mode of reactants addition i.e., when phenolic compounds were adsorbed first and then laccase was added (unpublished data). It should be mentioned that inorganic nature of solid support was not a necessary pre-condition for the polymeric HS formation. In our experiments on soil HA transformation in submerged culture of laccase-producing fungus *P. tigrinus*, polymerization of low molecular weight HA fractions into higher molecular weight products was observed on the mycelium surface (Zavarzina et al., unpublished data) (Fig. 10.5).

Although the possibility of HS formation by surface polymerization is not in doubt, the reaction mechanisms remain largely unclear. We can speculate that surface humus polymerization is a complicated heterophase process consisting of the following possible steps: (1) electrostatic substrate attraction to the surface; (2) enzyme-catalyzed substrate oxidation to free radicals at solid/solution interface; (3) adsorption of free-radical intermediates together with initial substrate on the mineral surface; (4) spontaneous polymerization/cross-coupling with polymer chain growth perpendicular to the surface (Fig. 10.6). It is important to underline that the concept of surface polymerization implies that polymeric organic films are produced on the surface before polymerization in the solution bulk has started (Sapurina et al. 2003). However, further experiments are needed to define the reaction sequence at solid/solution interface leading to humic polymers formation. For example, it might be that at first substrate adsorption occurs and then its oxidation proceeds directly on the surface by immobilized enzyme molecules. In this case, adsorbed phenolic substrate should interact somehow with enzyme active site (substrate adsorption onto the enzyme molecule is required). The study of Wershaw and Pinckney (1980) apparently supports such pathway as they found that organic matter is often bound to mineral surfaces by amino acids or peptides.

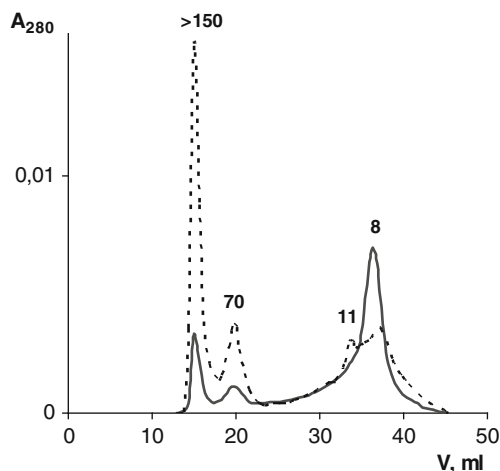


Fig. 10.5 Gel-chromatograms of HA, extracted from mycelium of *Panus tigrinus* on days 9 (solid line) and 12 (dashed line) of submerged cultivation of the fungus in presence of dissolved soil HA. The increase in the amounts of high MW fractions was due to surface oxidation and polymerization of HA by laccase, produced by the fungus. Bold numbers represent MWs in kDa. Column 1×60 cm, Sephadex G-100 gel, elution by 0.025M Tris-HCl buffer (pH 8.2) with 0.05M NaCl, 0.1% SDS, and 0.02% NaN₃ at a flow rate 3 ml h⁻¹

Perpendicular orientation of the polymeric product to the mineral surface is an important condition of surface polymerization process, experimentally confirmed for e.g., polyaniline formation (Sapurina et al. 2002). Vertical orientation of polymers should have a positive effect both on the reaction thermodynamics (e.g., Gerstner et al. 1994) and on enzyme activity, because there is a high probability that at least some enzyme molecules would not be inactivated by the growing polymer (as in the case of planar polymer orientation). The concept of vertical orientation of humic polymers during surface polymerization is in good agreement with the models of organic matter organization on the natural solid surfaces. Recent research has shown that aluminosilicate sediments with the loadings of organic matter <3 mg C m⁻² have less than 15% of their surface coated (Arnarson and Keil 2001). It was experimentally confirmed that distribution of organic (humic) material on the mineral surface is not uniform and it is organized in discrete spots (patches) with some vertical extension; the coating increases in thickness while retaining practically the same surface coverage (Wang and Xing 2005).

The recent concept of zonal self-organization of adsorbing molecules on mineral surfaces (Wershaw, 1993; Guggenberger and Kaiser 2003; Kleber et al. 2007) can provide an explanation for the effective surface polymerization of monomers when first precursors are adsorbed and then the enzyme is added (see above). It is considered that with increasing surface loading, an increasing portion of the sorbing molecules do not attach to mineral surface, but form organic multilayers as a result of hydrophobic interactions or bridging by polyvalent cations (Guggenberger and Kaiser 2003). The first layer (contact zone) may be more compact due to direct

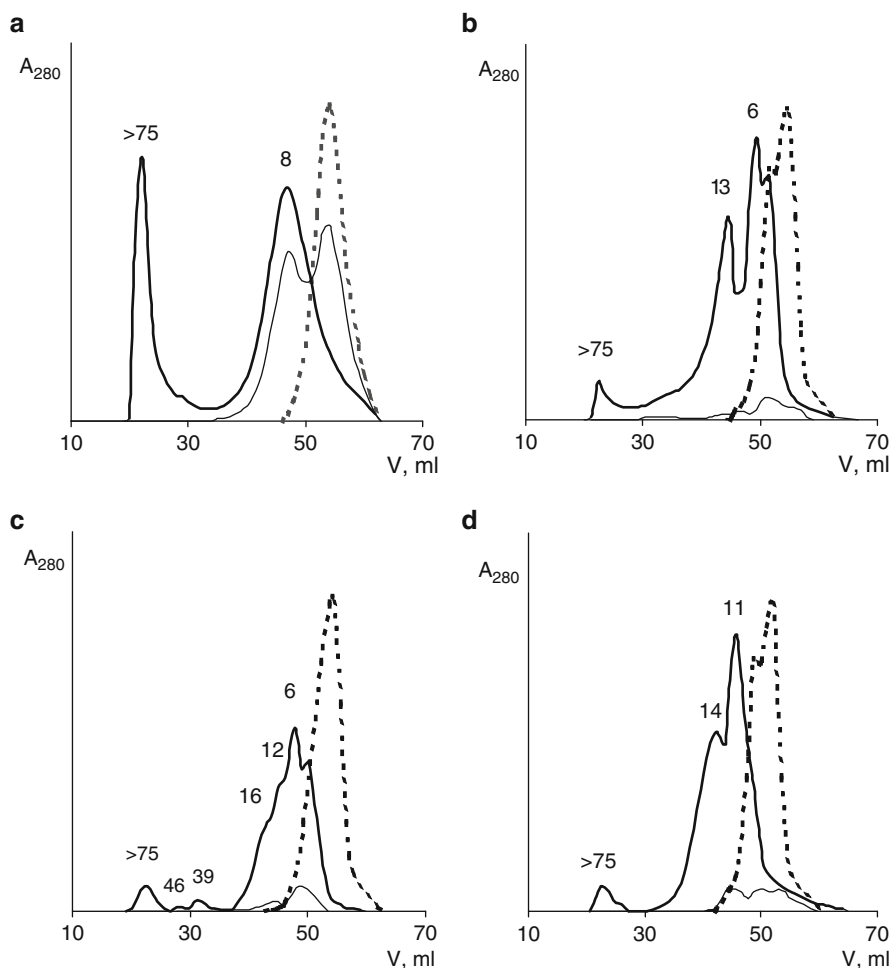


Fig. 10.6 Molecular weight distribution patterns of synthetic humic substances formed on the surface of clay minerals in the presence (*bold line*) or absence (*thin line*) of immobilized laccase: (a) hydroxyaluminum-kaolinite; (b) kaolinite; (c) montmorillonite; (d) illite. *Bold numbers* represent molecular weights in kDa. Gel chromatogram of precursor mixture is shown in dashed line. Column 1×60 cm, Sephadex G-75 gel, elution by 0.025 M Tris-HCl buffer (pH 8.2) with 0.05 M NaCl, 0.1% SDS, and 0.02% NaN_3 at a flow rate 8 ml h^{-1}

electrostatic attraction to the mineral surface; the components of second layer (hydrophobic zone) are more dynamic and can exchange with soil solution although being retained with considerable force, while molecules in the outer region of hydrophobic zone are loosely retained by cation bridging or hydrogen bonding and form kinetic zone (Kleber et al. 2007). Such organization of phenolic and nitrogenous precursors upon adsorption should enable enzyme and O_2 diffusion within organic “brushes” resulting in the oxidative cross-coupling between the components of contact, hydrophobic, and kinetic zones.

Oxidative polymerization of soluble precursors by immobilized enzymes at solid/solution interface can serve as good explanation for the existence of high molecular weight HA-like coatings on mineral particles. The reaction proceeds despite the reduced catalytic efficiency (lower V_{\max} values) and substrate affinity (higher K_m constants) of immobilized enzymes (Nannipieri and Gianfreda 1998). The question arises as to whether presence of biotic catalyst is obligatory or polymer formation on the mineral surface can occur abiotically as well.

10.2.2 Abiotic Heterogeneous Catalysis

Numerous studies have shown that various inorganic soil constituents, such as metal oxides (Shindo and Huang 1984; Lehmann et al. 1986; McBride 1987), hydroxides (Liu and Huang 2002), clay minerals (Wang et al. 1978), and even primary minerals (Shindo and Huang 1985) possess oxidative activities and can catalyze transformation of phenolic compounds into humic-like substances. Smectites were even able to catalyze Maillard reaction (Gonzalez and Laird 2004). Manganese (IV) oxides, such as common soil mineral birnessite (δ - MnO_2) are considered as the most powerful oxidants of phenolic compounds. The catalytic power of Fe (III) oxyhydroxides was much lower (Shindo and Huang 1984). As for the clay minerals, montmorillonite and illite were found to be better catalysts than kaolinite because their active sites were located on the planar surfaces and not on crystal edges as in case of kaolinite (Wang and Li 1977). Among the primary minerals studied, the oxidative power of tephroite (Mn-bearing silicate) was the greatest, followed by actinolite, hornblende, fayalite, augite, biotite, and muscovite = orthoclase = microcline = quartz (Shindo and Huang 1985). In general, the presence of transition metals (especially Mn) on the mineral surface or in the crystal lattice is required for efficient abiotic catalysis (Wang et al. 1986; Huang 2000). It was found that the nature of phenolic substrates affected the rate of their transformation into HA-like products. Polyphenols and polyhydroxyphenolic acids with para- and ortho-OH groups were more rapidly converted into HAs by Mn oxides than phenolic compounds with meta-oriented OH groups (Pohlman and McColl 1989; Shindo 1990). Any electron-attracting carboxyl group substituted on the ring reduced the polymerization rate while electron-releasing methyl group increased the rate (Wang et al. 1983).

The widely accepted mechanism of phenol oxidation by soil metal oxides is precipitation polymerization (Stone and Morgan 1984; McBride 1987), which involves the following steps: (1) binding of the organic molecule to the surface via phenolic or carboxylic groups; (2) electron transfer from the adsorbed organic to the oxide (surface oxidation); (3) release of oxidized molecule and reduced metal into solution due to dissociation of the complex; (4) under the aerobic conditions, the reduced metal is quickly re-oxidized, while semiquinones and quinones produced from phenolic substrate oxidation undergo spontaneous polymerization in aqueous phase with subsequent adsorption of the polymeric product on the mineral.

An alternative mechanism has been proposed recently for catechol polymerization, which includes: (1) heterogeneous catechol oxidation on metal oxide surface leading to release of reduced metal in solution; (2) immediate complexation of reduced metal ions by dissolved catechol; (3) homogeneous oxidation of metal–catechol complexes by dissolved oxygen, resulting in the formation of insoluble polymers (Colarieti et al. 2006). If no metal–organic complex dissociation occurs, the insoluble organo-mineral compounds are formed (Wang et al. 1978). In both cases, precursor surface complex formation is prerequisite for the electron transfer and is a rate-limiting step, while the electron transfer within surface complex is rapid (Matocha et al. 2001).

The overall difficulty with analyzing the results of abiotic catalysis in terms of polymeric HS formation is that the molecular weights of reaction products have rarely been measured. Dark-colored compounds were designated as polymeric HS on the basis of spectroscopy data and product insolubility either in aqueous (Liu and Huang 2002) or in the acidic medium (e.g., Wang et al. 1978; Shindo and Huang 1984, 1985; Shindo 1990). Even if we assume that these precipitates were polymeric, their formation under natural soil conditions is hardly possible because very high precursor concentrations ($1\text{--}10\text{ mg ml}^{-1}$) and long reaction periods (2–14 days) were used to produce them under “ideal” laboratory conditions. When mass spectrometry and high pressure liquid chromatography were used to analyze molecular weights of soluble and insoluble products of abiotic oxidation, it was found that they were mostly oligomeric by nature (Lehmann and Cheng 1988; Naidja et al. 1998).

Comparative studies on synthesis of humic-like substances using biotic and abiotic catalysts have shown that enzymatic oxidation of phenolic precursors was substantially more rapid than abiotic reaction (Pal et al. 1994; Bollag et al. 1995; Naidja and Huang 2002; Ahn et al. 2006). Molecular weights and the degree of aromatic ring condensation were higher in the products of biotic catalysis (Naidja et al. 1998). Interestingly, it was found that heterogeneous catalysis of liquid substrates by solid abiotic catalysts obeyed the Henri–Michaelis–Menten kinetic model (Naidja and Huang 2002). Determination of the kinetic constants for abiotic catalysis allowed direct quantitative comparison of catalytic efficiency between the enzyme (tyrosinase) and the mineral oxide (birnessite). It was found that while the V_{\max} of tyrosinase was 2.5–4 times higher than that for birnessite, the turnover frequency (k_{cat}) and the efficiency ($k_{\text{cat}}/K_{\text{m}}$) of the enzyme were three to four orders of magnitude higher than those of the mineral oxide (Naidja and Huang 2002). Higher efficiency of enzymes as oxidative agents than soil minerals is attributed to the ability of continuous oxidation of a substrate (Pal et al. 1994) while inorganic soil constituents can lose their oxidizing ability quite rapidly. The number of active sites on the mineral surface considerably decreases upon mineral aggregation or adsorption of reaction products on mineral surface (Lehmann and Cheng 1988). The abiotic reaction can be thus terminated once organic coating on the mineral surface has been produced.

It can be thus concluded that abiotic catalysis cannot lead to highly polymeric products formation ($>10\text{ kDa}$), at least at solute concentrations close to those in

natural soil environment. Absence of polymer formation is not only the result of slow kinetics of the process but also a result of active sites inactivation by reaction products. Nevertheless, abiotic oxidation route can be considered as one of the important mechanisms of organic matter stabilization in soils, especially at primary stages of soil development.

10.3 Conclusions

The experimental data summarized in this chapter allow us to conclude that high molecular weight HAs and their adsorption complexes in mineral soil horizons can originate from surface polymerization of low molecular weight precursor molecules on minerals in the presence of enzymatic catalysts. Polymerization at the surface precedes precipitation polymerization in the solution bulk. The positive effect of interfaces on the polymerization process lies in the energetics of the reaction: substrate concentration at the solution/solid interface is higher than that in dilute solution bulk, making polymerization reaction thermodynamically favorable. The positive effect of enzymatic catalysts on surface polymerization process lies in the reaction kinetics, resulting in accelerated rates of polymer formation in comparison to enzyme-free systems. Surface polymerization is complicated heterophasic multistep process, which is likely to include adsorption/oxidation and polymerization/cross-coupling steps. The concept of surface polymerization implies vertical polymer chain growth on the solid support surface, which is in good agreement with models of humic matter organization at natural solid phases (Kleber et al. 2007). It is very likely that surface polymerization produce polymeric organo-mineral complexes with different strengths of organic component binding to the mineral surface. Molecules attached directly to the mineral surface form humin-like structures upon oxidation. Organic compounds from the next layers can be extracted as HA fraction after oxidative polymerization of adsorbed organic molecules occurred. As a general conclusion, heterophase synthesis of HAs at mineral surfaces by immobilized enzymes may have wide application to soil systems given that enzymes and their substrates are commonly bound to mineral surfaces, and substrate concentrations in the aqueous soil phase are extremely low. Synthesis of humic substances on solid–solution interfaces can be particularly important in soils of cold and temperate humid climate, rich in Al and Fe oxyhydroxides. The mechanisms of heterophase reactions of humus synthesis remain largely unclear and need to be elucidated in future research. The effects of abiotic catalysts on the heterogeneous enzymatic catalysts should be also defined. Experimental evidence exists that enzyme-catalyzed reaction can be inhibited in presence of strong inorganic oxidants (such as birnessite) due to enzyme deactivation by humic-like compounds produced by the mineral (Ahn et al. 2006).

Acknowledgments The work was supported by the Programme No. 15 of the Presidium of the Russian Academy of Sciences “Origin of the Biosphere and Evolution of Geobiological systems”

and Russian Foundation for Fundamental Research grant No. 09-04-00570. The author expresses her sincere gratefulness to Prof. Richard P. Beckett for English revision and to Dr. Alexander A. Lisov for the assistance in preparation of Figs. 10.1, 10.3, and 10.4.

References

- Ahn MY, Martinez CE, Archibald DD, Zimmerman AR, Bollag JM, Dec J (2006) Transformation of catechol in presence of a laccase and birnessite. *Soil Biol Biochem* 38:1015–1020
- Ahn MY, Zimmerman AR, Martinez CE, Archibald DD, Bollag JM, Dec J (2007) Characteristics of *Trametes villosa* laccase adsorbed on aluminum hydroxide. *Enzym Microb Technol* 41:141–148
- Allison SD, Jastrow JD (2006) Activities of extracellular enzymes in physically isolated fractions of restored grassland soils. *Soil Biol Biochem* 38:3245–3256
- Amanson TS, Keil RG (2001) Organic-mineral interactions in marine sediments studied using density fractionation and X-ray photoelectron spectroscopy. *Org Geochem* 32: 1401–1415
- Batjes NH (1996) Total carbon and nitrogen in the soils of the world. *Eur J Soil Sci* 47:151–163
- Bollag JM, Minard RD, Liu SY (1983) Cross-linkage between anilines and phenolic humus constituents. *Environ Sci Technol* 17:72–80
- Bollag JM, Meyers C, Pal S, Huang PM (1995) The role of abiotic and biotic catalysts in the transformation of phenolic compounds. In: Huang PM, Berthelin J, Bollag JM, McGill WB, Page AL (eds) Environmental impact of soil component interactions, vol 1. CRS/Lewis, Boca Raton, FL, pp 299–310
- Bollag JM, Dec J, Huang PM (1998) Formation mechanisms of complex organic structures in soil habitats. *Adv Agron* 63:237–265
- Boufi S, Gandini A (2002) Formation of polymeric films on cellulosic surfaces by admicellar polymerization. *Cellulose* 8:303–312
- Cecchi AM, Koskinen WC, Cheng HH, Haider K (2004) Sorption-desorption of phenolic acids as affected by soil properties. *Biol Fertil Soils* 39:235–242
- Christensen BT (2001) Physical fractionation of soil and structural and functional complexity in organic matter turnover. *Eur Soil Sci* 52:345–353
- Colarieti ML, Toscano G, Ardi MR, Greco G (2006) Abiotic oxidation of catechol by soil metal oxides. *J Hazard Mater* 134:161–168
- Criquet S, Farnet AM, Tagger S, Le Petit J (2000) Annual variations of phenoloxidase activities in an evergreen oak litter: influence of certain biotic and abiotic factors. *Soil Biol Biochem* 32:1505–1513
- Dalton BR, Blum U, Weed SB (1989) Plant phenolic acids in soils: sorption of ferulic acid by soil and soil components sterilized by different techniques. *Soil Biol Biochem* 21: 1011–1018
- Danielewicz-Ferchmin I, Ferchmin AR (2004) Water at ions, biomolecules and charged surfaces. *Phys Chem Liq* 42:1–36
- Di Nardo C, Cinquegrana A, Papa S, Fuggi A, Fioretto A (2004) Laccase and peroxidase isoenzymes during leaf litter decomposition of *Quercus ilex* in a Mediterranean ecosystem. *Soil Biol Biochem* 36:1539–1544
- Fedorova S, Stejskal J (2002) Surface and precipitation polymerization of aniline. *Langmuir* 18:5630–5632
- Fenner N, Freeman C, Reynolds B (2005) Hydrological effects on the diversity of phenolic degrading bacteria in a peatland: implications for carbon cycling. *Soil Biol Biochem* 37:1277–1287
- Flaig W (1966) The chemistry of humic substances. In: The use of isotopes in soil organic matter studies, Report of FAO/IAEA Technical Meeting. Pergamon, New York, pp 103–127

- Gallet C, Pellissier F (1997) Phenolic compounds in natural solutions of the coniferous forest. *J Chem Ecol* 23:2401–2411
- Gerstner JA, Bell JA, Cramer SM (1994) Gibbs free energy of adsorption of biomolecules in ion-exchange systems. *Biophys Chem* 52:97–106
- Gonzalez JM, Laird DA (2004) Role of smectites and Al-substituted goethites in the catalytic condensation of arginine and glucose. *Clays Clay Miner* 52:443–450
- Gramss G, Voigt KD, Kirsche B (1998) Oxidoreductase enzymes liberated by plant roots and their effects on soil humic material. *Chemosphere* 38:1481–1494
- Guggenberger G, Kaiser K (2003) Dissolved organic matter in soil: challenging the paradigm of sorptive preservation. *Geoderma* 113:293–310
- Huang PM (2000) Abiotic catalysis. In: Summer ME (ed) *Handbook of soil science*. CRC Press LLC, Boca Raton, FL, pp 303–334
- Huang Q, Weber WJ (2004) Peroxidase-catalyzed coupling of phenol in the presence of model inorganic and organic solid phases. *Environ Sci Technol* 38:5238–5245
- Huang PM, Wang MK, Kampf N, Schulze DG (2002a) Aluminum hydroxides. In: Dixon JB, Schulze DG (eds) *Soil mineralogy with environmental applications*. Soil Science Society of America, Madison, WI, USA, pp 261–289
- Huang Q, Selig H, Weber WJ (2002b) Peroxidase-catalyzed oxidative coupling of phenols in the presence of geosorbents: rates of non-extractable product formation. *Environ Sci Technol* 36:596–602
- Kalbitz K, Solinger S, Park JH, Michalzik B, Matzner E (2000) Controls on the dynamics of dissolved organic matter in soils: a review. *Soil Sci* 165:277–304
- Klavinš M (1997) *Aquatic humic substances: characterization, structure and genesis*. University of Latvia, Riga, 234 p
- Kleber M, Sollins S, Sutton RA (2007) A conceptual model of organo-mineral interactions in soils: self-assembly of organic molecular fragments into multilayered structures on mineral surfaces. *Biogeochem* 85:9–24
- Kononova MM (1966) *Soil organic matter*. Pergamon, Oxford
- Kuvaeva YV (1980) The content and composition of phenolic acids in some soils of non-chernozemic zone. *Sov Soil Sci* 1:97–106
- Lambert JF (2008) Adsorption and polymerization of amino acids on mineral surfaces: a review. *Orig Life Evol Biosph* 38:211–242
- Lehmann RG, Cheng HH (1988) Reactivity of phenolic acids in soil and formation of oxidation products. *Soil Sci Soc Am J* 52:1304–1309
- Lehmann RG, Cheng HH, Harsh JB (1986) Oxidation of phenolic acids by soil iron and manganese oxides. *Soil Sci Soc Am J* 51:352–356
- Leontievsky AA, Myasoedova NM, Baskunov BP, Pozdnyakova NN, Vares T, Kalkkinen N et al (1999) Reactions of blue and yellow fungal laccases with lignin model compounds. *Biochemistry (Moscow)* 64:1150–1156
- Liu C, Huang PM (2002) Role of hydroxy-aluminosilicate ions (proto-imogolite sol) in the formation of humic substances. *Org Geochem* 33:295–305
- Liu SY, Freyer AJ, Minard RD, Bollag JM (1985) Enzyme-catalyzed complex formation of amino acid esters and phenolic humus constituents. *Soil Sci Soc Am J* 49:337–342
- Matocha CJ, Sparks DL, Amonette JE, Kukkadapu RK (2001) Kinetics and mechanism of birnessite reduction by catechol. *Soil Sci Soc Am J* 65:58–66
- McBride MB (1987) Adsorption and oxidation of phenolic compounds by iron and manganese oxides. *Soil Sci Soc Am J* 51:1466–1472
- Mikutta R, Kleber M, Torn MS, Jahn R (2006) Stabilization of soil organic matter: association with minerals or chemical recalcitrance? *Biogeochemistry* 77:25–56
- Naidja A, Huang PM (2002) Significance of the Henri-Michaelis-Menten theory in abiotic catalysis: catechol oxidation by δ -MnO₂. *Surface Sci* 506:243–249
- Naidja A, Huang PM, Bollag JM (1997) Activity of tyrosinase immobilized on hydroxyaluminum-montmorillonite complexes. *J Mol Catal A* 115:305–316

- Naidja A, Huang PM, Bollag JM (1998) Comparison of reaction products from the transformation of catechol catalyzed by birnessite or tyrosinase. *Soil Sci Soc Am J* 62:188–195
- Nannipieri P, Gianfreda L (1998) Kinetics of enzyme reactions in soil environments. In: Huang PM, Senesi N, Buffle J (eds) *Environmental particles – structure and surface reactions of soil particles*. Wiley, Chichester, pp 449–479
- Pal S, Bollag JM, Huang PM (1994) Role of abiotic and biotic catalysts in the transformation of phenolic compounds through oxidative coupling reactions. *Soil Biol Biochem* 26:813–820
- Perdue EM, Ritchie JD (2004) Dissolved organic matter in freshwaters. In: Holland HD, Turekian KK (eds) *Treatise on geochemistry*. Elsevier, Amsterdam, pp 273–318
- Pohlman AA, McColl JG (1989) Organic oxidation and manganese and organic mobilization in forest soils. *Soil Sci Soc Am J* 53:686–690
- Quiquampoix H, Servagent-Noinville S, Baron M (2002) Enzyme adsorption on soil mineral surfaces and consequences for the catalytic activity. In: Burns RG, Dick RP (eds) *Enzymes in the environment*. Marcel Dekker, New York, pp 285–306
- Rittstieg K, Suurnakki A, Suortti T, Kruus K, Guebitz G, Buchert J (2002) Investigations on the laccase-catalyzed polymerization of lignin model compounds using size-exclusion HPLC. *Enzyme Microb Technol* 31:403–410
- Sapurina I, Osadchev AY, Volchek BZ, Trchova M, Riede A, Stejskal J (2002) In-situ polymerized polyaniline films: 5. Brush-like chain ordering. *Synth Met* 129:29–37
- Sapurina I, Fedorova S, Stejskal J (2003) Surface polymerization and precipitation polymerization of aniline in presence of sodium tungstate. *Langmuir* 19:7413–7416
- Sarkar JM, Leonowicz A, Bollag JM (1989) Immobilization of enzymes on clays and soils. *Soil Biol Biochem* 21:223–230
- Schulze DG (2002) An introduction to soil mineralogy. In: Dixon JB, Schulze DG (eds) *Soil mineralogy with environmental applications*. Soil Science Society of America, Madison, WI, pp 1–36
- Shindo H (1990) Catalytic synthesis of humic acids from phenolic compounds by Mn(IV) oxide (birnessite). *Soil Sci Plant Nutr* 4:679–682
- Shindo H, Huang PM (1984) Catalytic effects of manganese (IV), iron (III), aluminum and silicon oxides on the formation of phenolic polymers. *Soil Sci Soc Am J* 48:927–934
- Shindo H, Huang PM (1985) Catalytic polymerization of hydroquinone by primary minerals. *Soil Sci* 139:505–511
- Sjogblad RD, Bollag JM (1981) Oxidative coupling of aromatic compounds by enzymes from soil microorganisms. In: Paul EA, Ladd JN (eds) *Soil biochemistry*, vol 5. Marcel Dekker, New York, pp 113–152
- Snajdr J, Valaskova V, Merhautova V, Herinkova J, Cajthaml T, Baldrian P (2008) Spatial variability of enzyme activities and microbial biomass in the upper layers of *Quercus petraea* forest soil. *Soil Biol Biochem* 40:2068–2075
- Stevenson FJ (1994) *Humus chemistry: genesis, composition, reactions*, 2nd edn. Wiley, New York
- Stone AT, Morgan JJ (1984) Reduction and dissolution of manganese (III) and manganese (IV) oxides by organics: 1. Reaction with hydroquinone. *Environ Sci Technol* 18:450–456
- Tietjen T, Wetzel RG (2003) Extracellular enzyme-clay mineral complexes: enzyme adsorption, alteration of enzyme activity and protection from photodegradation. *Aquat Ecol* 37:331–339
- Tipping E, Cooke D (1982) The effects of adsorbed humic substances on the surface charge of goethite (α -FeOOH) in freshwaters. *Geochim Cosmochim Acta* 48:75–80
- Toberman H, Evans CD, Freeman C, Fenner N, White M, Emmett BA, Artz RRE (2008) Summer drought effects upon soil and litter extracellular phenol oxidase activity and soluble carbon release in an upland Calluna heathland. *Soil Biol Biochem* 40:1519–1532
- Wang TSC, Li SW (1977) Clay minerals as heterogeneous catalysts in preparation of model humic substances. *Z Pflanzenernaehr Bodenkd* 140:669–676
- Wang K, Xing B (2005) Structural and sorption characteristics of adsorbed humic acid on clay minerals. *J Environ Qual* 34:342–349

- Wang TSC, Li SW, Ferng YL (1978) Catalytic polymerization of phenolic compounds by clay minerals. *Soil Sci* 126:15–21
- Wang TSC, Wang MC, Ferng YL, Huang PM (1983) Catalytic synthesis of humic substances by natural clays, silts and soils. *Soil Sci* 135:350–360
- Wang TSC, Huang PM, Chou CH, Chen JH (1986) The role of soil minerals in the abiotic polymerization of phenolic compounds and formation of humic substances. In: Huang PM, Schnitzer M (eds) *Interactions of soil minerals with natural organics and microbes*. Soil Science Society of America, Madison, WI, USA, pp 251–281
- Wershaw RL (1993) Model for humus in soils and sediments. *Environ Sci Technol* 27:814–816
- Wershaw RL, Pinckney DJ (1980) Isolation and characterization of clay-humic complexes. In: Baker RA (ed) *Contaminants and sediments*. Ann Arbor Science Publishers, Ann Arbor, Michigan, pp 207–219
- Yagudaeva EYu, Muidinov MR, Kapustin DV, Zubov VP (2007) Oxidative polymerization of aniline on the surface of insoluble solid poly(sulfo acids) as a method for the preparation of efficient biosorbents. *Russ Chem Bull, Int Edn* 56:1166–1173
- Zavarzina AG (2006a) A mineral support and biotic catalyst are essential in the formation of highly polymeric soil humic substances. *Eurasian Soil Sci* 39:48–53
- Zavarzina AG (2006b) Key role of mineral support and biotic catalyst in the formation of highly polymeric soil humic acids. In: *Proceedings of the 13th meeting of the international humic substances society*. Karlsruhe, Germany, July 30–Aug 4 2006, pp 605–608
- Zavarzina AG, Semenova TA, Kuznetsova AM, Pogozhev EYu (2007) Synthesis of humic-like substances on the mineral surfaces in presence of oxidases. In: *Proceedings International Conference “Humic substances in the biosphere.”* Moscow, Dec 19–21, 2007, pp 139–145 (in Russian)