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Characterization, transformation and application of humic substances – Review

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ABSTRACT

Humic substances are derived from Soil Organic Matter (SOM) which is playing a major role in agriculture. Quality, maturity, nativity assessment and activity of humus are determined by hydrophobic and hydrophilic (HB/HI) index, which is based on chemical composition of core structure of humic substances. Because till now there is no exact chemical formula or structure for HA and it is vary depend on source material, microbial populations, physical and chemical environment for humic substances formation, but in general it is composed of C,H, O and N. Important functions of Humic substances in environment are, it aids in nutrient solubilization from drought and compacted soils, nutrient uptake by plants, heavy metals immobilization, controlling soil erosion, improved multiplication of essential microorganisms (EM) for agriculture. This review introduces the basic properties, composition, chemical reactivity and characterization of HS with highlighting the need of innovative research for better fundamental understanding of the mechanisms, formation transformations and application of humic substances.

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KEYWORDS

Soil organic matter;
Humic acid;
Humic substances;
Humic acids application;
Soil fertility.

INTRODUCTION

The clear knowledge of soil and its vegetation of each respective area help us to use proper application of necessary supplements. Due to globalization farmers use chemicals in all areas of agriculture without its knowledge of usage, activity or its property in particular level, leads to become a cause for serious environmental problems (including bioaccumulation, biomagnifications, changes in biodiversity and climate changes) which ultimately ends in risk of people's health. The present situation before us is to take an immediate and intellectual step for the improvement of agriculture with correct

implementation of natural with chemical methods and thus improving/ not damaging the present environment. Human activities in immediate past and last ten years to soil causes changes in organic matter, type of biological influences over organic matter and available Vs bounded nutrients in the soil. These changes create impact in pH and nutrient cycling in the environment. In really soil OM is not a measurable soil component.

Soil organic matter (SOM) is not a measurable component and it is present in fertile soil (2-8%), neutral and alkaline soil (40-60% in the form of humic acid and humin) and acid leached soils (Petit). SOM encompass all OM fractions present in soil, including crop residues

(litter), plant residues in varying stages of decomposition, microbial biomass, dissolved OM and stable humus^[1]. Humus does not occur alone in soil, it is made up of humic substances composed of C, O & H. Humic substances include humic acids (HAs), fulvic acids (FAs) and humins. Humic acids are the end product of microbial degradation of plant and animal debris and are one of the most important constituents of fertile soils. Humic acid is defined as a portion of soil humus that is soluble in alkaline solution, but insoluble in acid solution, which is the form of organic matter that often is added to the soil to increase fertility.

SOURCES OF HUMIC ACID

Humic acids are present in soil (65-75%) composts vermicompost^[45], activated sludge^[62], sewage sludge^[9], urban waste compost, sugar cane filter cake, leonardite coal^[24], organic matter in low land rice soils^[48], rhizosphere soil^[45], organic soils or histosols^[1], wetland soils, natural wetland environments^[17], deep sea sediments^[40], estuaries and lakes^[6] and in other aquatic systems^[60].

FORMATION OF HUMIC ACID

Initial formation or formation of neo polymers

Humification or humus formation is described through composting process^[2]. Composting determines the increase in humic substance content. Monitoring of humification indirectly acts as a key factor to know the quality of composting. In early stage of this process oxygen rich compounds like polysaccharide (aliphatic C), lipids and proteins (called interference materials) are degraded to other oxygenated compounds^[2] particularly carboxylic and ester groups with an increase of aromatic structures suggest the partial formation of neo-humic polymers (humification)^[12]. While other, more oxidized, recalcitrant compounds are neo-formed in the course of humification^[2]. During composting the extensive degradation suffered by interference materials or organic compounds like lignin, cellulose, hemicelluloses and proteins which leads to release of great variety of simple organic compounds such as carbohydrates, amino acids, simple peptides and phenols of low structural complexity^[14]. This study is confirmed by analyzing soil using Tetramethylammonium Hydroxide Thermochemolysis-GC/MS (TMAH-GC/MS), in

which lignin derivatives were analyzed in different soil size fractions and the resulting data indicates that the soil fractions, >250- μm chromatogram reveals more lignin-derived compounds and their relative intensities are more pronounced than in <2- μm chromatogram. During humification, these structures are further transformed to aromatic humic-like structures, where lignin side chains are oxidized. Further Soumia et al. (2010) has explained through composting of activated sludge, in that on 135th day of composting aliphatic C content is decreased to 30.1 and high content of aromatic C to 45.6 and carboxyl C content of 24.3, and in FTIR and ¹³C NMR analysis shows aromatic C/aliphatic C ratio decreased at initial times and then increased, which showed the initial phase of intense biodegradation of the large amounts of aliphatic compounds, even though some imbalances during composting was there. These aliphatic structures then decomposed during last step of composting with the help of microbial communities proliferating after the death of original ones to the synthesis of new, more humified materials^[29]. Kogel-Knabner (2002) also suggests that these subsequently formed structures of aliphatics could either be released through degradation of lignins or could originate from microbial tissues.

Formation of core structures

Aliphatic and aromatic constituents form the core structure of humic substances or called true humic acids^[2] or core humic acids and these are derived from various organic sources which are involved in degradation reactions^[14]. The presence of non-humic substances in humic acid from soil are amino acids, hydrolysable phenolic acid, hexose and uronic acid as mixtures, which smeared the spectral data of HA materials^[32]. The evolution of core humic acid from municipal sewage waste (MSW) composting showed that the nucleus did not change its chemical structure during the process that it differed from the humic acids only in the presence of the organic molecules (interference materials) that were eliminated or rapidly degraded during composting^[14] whereas, the humic material remains constant^[1]. Researchers believe that the chemical and biochemical reactions that occur during humification transform the organic matter through stabilization of aromatic structures, leading to a relative enrichment of aromatic carbon and selective preservation of alkyl carbon of soil

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biopolymers resistant to decomposition, such as cutins and suberins.

Structural conformation and stability

Humic acid's chemical stability and interaction with environment were determined by hydrophobicity nature^[52]. Insam (1996) has stated that the covalent polymerization of HS is by random condensation and oxidation processes of simple monomeric units into macromolecular polymers, and they are in chain (linear or branched), that causes its resistance to microbial degradation and consequent long residence time for soil humic components. The relatively large abundance of aromatic and alkyl C in all HA indicated a general major contribution of hydrophobic carbon (HB) to humic composition and these hydrophobicity cause the strict association of aromatic and alkyl C with clay and silt particles^[65]. HS are relatively small molecules held together by weak interaction forces, forming a supramolecular structure^[53]. The supramolecular organizations are stabilized only by weak forces such as dispersive hydrophobic interactions (van der Waals, π - π , and CH- π) and hydrogen bonds which is being progressively more important at low pH values^[51]. In solution HS are macromolecules and assume random coil conformations, become micelles or "pseudomicellar" structures. Organic acids in soil solution induce conformational changes in hydrophobic domains of HA^[54].

Aromaticity index for determining nativity

The hydrodynamic [hydrophilic (HI) or hydrophobic (HB)] volume of humic molecules will determine the source from which humus is formed. The aromaticity index (aromatic and phenolic carbons abundance) of HAs of oxidized coal is more than lignite coal, determine by HI/HB index and higher values for this index is highly hydrophobic than others and lower values is higher hydrophilic nature of HA^[15]. Coal-derived humic acid distinguished from humic acid derived from natural sources by the fact that it contains a higher aromaticity and a higher ratio of phenolic to carboxylic groups in the ratio of about 1,46:1,08 respectively^[19].

PROPERTIES OF HUMIC ACID

Molecular size

Molecular size of humic acid considered to be a

function of the strength of hydrophobic interactions with bioactive molecules and hydration radius of the several different heterogeneous molecules associated in humic substances^[61]. In general alkyl components account for the largest molecular dimensions of HA^[52] and their strong reciprocal hydrophobic interactions leading to large molecular aggregates, although carbohydrate-cellulose C is an important component of HA from stabilized compost material^[11,63,64]. The presence of auxin in HA was also reported^[10].

Molecular size determination indirectly evaluate the chemical groups present in the HA and it can be investigated through elemental analysis. The elemental composition and atomic ratios (carbon distribution) of HA isolates can be seen through ¹³C-CPMAS-NMR spectra. In particular the summation of particular chemical shift region in ppm will show the important nature of HA characterization. They are summation of [(162-110)/(0-162) * 100] for AD (aromaticity degree), [(162-110) + (46-0)] for HB (hydrophobic C) and [(185-162) + (90-65) + (65-46)] for HI (hydrophilic C). Pseudo-2D DOSY technique is particularly suitable for complex samples such as HA because it provides a direct correlation of translational diffusion (D) with the chemical shift in the second dimension. Through this evaluation for three different NMR spectral regions will tell the chemical nature of HAs. (i) 9.0-5.0 ppm for protons on unsaturated carbons, nitrogen hetero atoms and amides; (ii) 4.4-2.9 ppm for protons on CH₃, CH₂ or CH carbons directly bonded to O, N or carbohydrates and (iii) 2.9-0.0 ppm for alkyl protons, including those on alkyl carbons that are two or more carbons away from aromatic rings or polar functional groups^[20].

Solubility

Humic acids dissolve in alkaline solutions (pH 7-8 and above) are called as mobile HA (MHA) and become precipitate in acidic pH (1-2). But water scientists consider that the precipitates forms at pH 2 to be HAs because non humic substances like some proteins also get precipitated under similar conditions, and so the precipitates at pH 1 might be considered as the HA fraction^[61].

Hydrodynamic size of a humic material rich in polar components (hydrophilic) will be kept small when dissolved in aqueous solutions due to stabilization exerted by hydration of water, conversely, more hydrophobic

materials self-associate in large dimensions and become separated from water, thereby decreasing the total free energy of the dissolved system^[59,69].

The solvation or soluble nature, polarity, cation exchange capacity (CEC) and diffusion behaviour of humic acid depends on the abundance of polar (hydrophilic) and apolar (hydrophobic) C in humic acid structure, was evaluated by HI/HB ratio (This also depends on topological and physiological arrangements/interactions/ bonding (of core structure) of HA^[30]). This hydrophobicity nature of HA also plays a role in sorption of bioactive molecules in the environment. The larger the potential hydrophobic incorporation of bioactive molecules (e.g., auxin like structures, some enzymes like protease) in humic structure tends to the larger chemical diversity of such bioactive molecules (polar molecules) and the more diverse their bioactivity^[50]. It was previously postulated that the hydrophobic humic components derived from plant degradation and microbial activity are able to randomly incorporate more polar molecules and hence protect them against degradation^[30].

Humic acid aging or maturity

Study on the age of formed humus will explain its importance to know the type and amount of humic components presence in humus or humic acids. With increasing humification, the color of the HA typically darkens from yellowish-brown to brown to nearly black^[36]. Ve et al. (2004)^[48] were studied the mineralization kinetics of soil organic matter in lowland rice soils by comparing the mobile humic acid (MHA) with calcium humate (CaHA), and said that MHA is a freshly formed humic acid in small mass, formed after puddling of land before cropping, has higher N concentration, lower C concentration and C/N ratio, smaller E_4 value than CaHA indicating the humified age of CaHA. They also reported that Generally MHAs of different soils in dry powder state were brown, dark-reddish brown and CaHA fraction was darker than the MHA fraction and CaHA fraction would be better protected against microbial degradation through its stabilization by polyvalent cations.

TOOLS FOR HUMIC ACID CHARACTERIZATION

Humic acid characterization can be performed by

several tools. Many numbers of researches has been carried out all over the world using various techniques. Each has its advantage over humic acid study, the tools used are primarily UV spectrum, UV deconvolution, followed by FTIR, NMR, ESR, CP-MAS, GC-MS, chromatography, SEM, TEM, Radio labeling, AFM, thermal imaging, ultra filtration technique etc for concentrated characterization^[22].

Techniques and its application for HA study

The first indirect evaluation of HA aromaticity was undertaken by Kononova (1961) via the E_4/E_6 ratio using UV spectrophotometer. This ratio is between the absorbance at 465 and 665 nm. In this as the E_4/E_6 ratio drops this indirectly indicates the rise in aromaticity together with humification for organic matter stabilization. Aromatic structure can be indirectly measured by the presence of C/H atomic ratio, because the measurement of C/H atomic ratio in humic acid could be a ratio of the alkyl C and the conjugated C linked to aromatic systems. Sugar cane filter cake contain relatively low C/H atomic ratio, HAs from urban waste compost and sewage sludge usually exhibit higher H/C ratio. If enriched with aromatic carbon means there would be a drop in H/C ratio.

In accordance to this, Chen et al. (1977) reported that this ratio also predicts the size of the humic substances in the form of molecular weight. Ultraviolet (UV) and visible (Vis) absorption spectra technique is used for study of aromatic carboxyl and phenolic groups and C=C systems^[37]. UV spectroscopy can confirms the maturity state of compost e.g. the absorbance around 250-300 nm shows the presence of aromatic groupings, this is a major part in HA structure and formed in matured compost through "secondary synthesis" by microorganisms. UV spectroscopy also represents absorbance at low range of UV wavelengths for mineralization phenomenon^[22].

NMR techniques are useful for quantification study, and to determine the structural units in HS like carbon atoms in carbohydrates, aromatic rings, carboxylic acids^[30,73]. The degree of condensation of aromatic rings in humic acid can be measured by NMR CP/MAS ¹³C techniques with polar dephasing (DD) measurements^[57].

Fourier Transform Infrared Spectroscopy (FTIR) are useful for qualitative study^[42] and provides information about the stretching and deformation vibrations

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of chemical bonds between atoms showing electric dipole moment characterized by non-zero value. It is possible to differentiate the source and the humification condition of the organic matter through FTIR spectrum. FTIR spectrum of sugar cane filter cake HS has relatively low degree of humification, such as FA (fulvic acid), has a spectrum without CH_3 , CH_2 and CH absorption bands (generally located around $2900\text{--}2850\text{ cm}^{-1}$) and the presence of wide and diffuse absorption band at 1100 cm^{-1} is characteristic of low-humified HS.

The growth promoters and other organic modifiers make weak association with humic molecules this is confirmed by peaks obtained for eluting solutions in HPSEC^[11]. The presence of auxin in HA was detected by different methods including immunoassays or gas chromatography – mass spectrometry^[10].

APPLICATION PROPERTIES OF HUMIC ACID IN AGRICULTURE

Humic acid is mainly used as plant growth substance, but other newer studies and approaches are emerging on humic acid for its better utility and impact in environment.

Humic acid as fertilizers

Humic acids can be a complement to synthetic or organic fertilizers and in some instances, fertilization can be eliminated entirely if sufficient organic material is present and the soil can become self sustaining through microbial processes and humus production, because of their ability to chelate micronutrients, thus increasing their bio-availability^[34]. The effectiveness of HA over plant growth (metabolic stimulation) is based on low molecular size HS (LMS-HS)^[71] and that could have large content of aromatic, carboxyl and phenolic carbons^[55].

The successful application of organic fertilizers in organic farming is based on the presence of humic matter. Fernandez et al. (1996)^[24] pointed out that under field conditions, foliar application of leonardite extracts stimulated the shoot growth and promoted the accumulation of K, B, Mg, Ca, and Fe in leaves. The reason for HA activity over plant growth were confirmed by the research on Maize seeds (*Z. mays* L. var. DK 585) by using different molecular fractions of HA for enzymatic cycle of respiration and suggested that the stimulation of plant metabolism may be more precisely

attributed to specific classes of compounds in humic substances or humic structures^[46].

In soil application of 2 g humus (70% w/w, pH 5.17, EC: 4.80 mS/cm) /kg soil to plants (corn) increase dry weight and nutrient uptake (especially N) but the results became negative for 4g humus/kg of soil and in foliar application of humic acid (humic acid: 15% w/v, pH 10.66, EC: 28.8 mS/cm) the highest dry weight and nutrients uptake were obtained with 0.1% dose and decreased with 0.2% but the amounts except for Fe, Cu, and Mn were found higher than in the control^[34]. This is because application of very high doses of humic acids is less effective^[38], but Cooper et al. (1998)^[18] applied humic substances for creeping bent grass in sand culture at rates of 100, 200 and 300 mg/l and found that the rate of application did not have any effect on the plant growth. Regarding these Pavlikova et al. (1997)^[49], also clearly indicated in their study by using humic acid in the form of potassium humate even at high concentration (20 mg/L) as foliar spray which did not affect the yield of crop.

Humic acid in contact with root system

Lu-lakis and Petras (1995) have stated that water uptake increases nutrient absorbance by the roots in the presence of humic acid, which enhances the development of lateral roots. HA fractions from earthworm compost was observed to show greater root size, more branched roots, root hairs with larger surface area and stimulation of H^+ -ATPase activity in maize plants^[45]. Increase in root fresh weight (30.1%) and root dry weight (56.6%) was examined in broad bean (*Vicia faba* L.) plant, this is due to the increased uptakement of K^+ and Na^+ (111.4% and 86.4% respectively) with the help of HA. Micronutrients Ca^{2+} , Fe^{3+} and Mn^{2+} in root cells of HA treated plants seem to be increased due to membrane permeability and transport which is caused by activation of voltage-dependent cation channels or voltage-independent cation channels (VIC) by Na^+ which enters root cells and increase lateral root development and total root bio-mass. But a high Na content in soil solution has also an antagonistic effect on the uptake of Ca and Mg^[8].

Humic substances may interact with the phospholipid structures of the cell membranes and react as carriers for nutrients^[34]. The surface activity of humic substances in plant surface is due to the presence of both

hydrophilic and hydrophobic sites^[16].

Humic acids having carboxylic and phenolic groups will enhance the absorbance capacity of nutrients in roots by increasing H⁺-ATPase activity in the root cells^[10]. Muscolo et al. (2007)^[45] postulated that the interaction between root system and humic matter is possible in the rhizosphere when humic molecules in the soil solution are small enough to flow into the apoplast and reach the plasma membrane. Simple organic acids exuded by plant roots and microbes^[50,51] help for releasing auxin like molecule from humic structure which could not be done, if HS structure is damaged by chemical modification or environmental factors. This leads to inactivation of the cell receptors in plasma membrane by H⁺-ATPase activity for the regulation of cytoplasmic pH and the activation of cell wall-losing enzymes and proteins through acidification of apoplast in plant roots^[67]. Humic structure from vermicompost was chemically modified using different acidic, basic and methyl compounds and the altered molecular weight humic acids were used for maize plant growth study and the study states that there was no relationship between bioactivity and molecular size of humic acid, but hydrophobic index appear to play an important role in the bioactivity of the modified humic matter.

Microbial activity on humic substances

Bacteria can use humic substances as organic electron acceptors and capable of reducing humic substance^[40]. According to Scott et al. 1998^[60] the electron accepting capacity of humic substances extracted from soils was much greater than dissolved humic substances of aquatic systems. Humic acid extracted from wetland soils and incubated in an anaerobic chamber showed the final ratio of CO₂:CH₄ >2, suggesting that humic acids may strongly limit the production of CH₄ by serving as thermodynamically favorable organic electron acceptors or terminal electron acceptor (TEMs). Humic substance analog anthraquinone-2, 6-disulfonate (AQDS) has been isolated from natural wetland environments and has reported that this analogue acts as TEMs^[17]. Jason et al. 2009, hypothesize that the chemical makeup of humic substances extracted from different wetland soils causes different range of ratios in CO₂:CH₄ production.

Humic substances are susceptible to microbial deg-

radation in the environment. The rates of bacterial degradation of HS may vary with age, source materials and bacterial community composition. The uptake of HS in living cells (*Escherichia coli*) and plants (sprouts and root system of *Triticum aestivum*) at different concentrations is demonstrated by radio labeling of humic acid with tritium^[6]. The adsorption capacity of HS to biological surfaces or any organic or inorganic materials will increase with increased H/C atomic ratio. Thurman (1985) said that the structural complexity of HS for microbial degradation which typically contain an aromatic core, aliphatic side chains, carboxyl groups and phenolic hydroxyl groups among other chemical moieties and based on this Esham et al. (2000)^[23] were categorized their bacterial cultures in three major phylogenetic groups (α -Proteobacteria, γ -Proteobacteria and Gram -positive bacteria) which were capable of growth in enrichment culture media with estuarine HS as the sole carbon source.

(Moran and Hodson, 1994; 1990) have reported that utilization of HS containing recalcitrant compounds become more complex material to microbial mineralization in low rates of [¹⁴C] in HS and low rates mineralization also reported for natural humic substances from freshwater and marine wetlands. Humic acids fraction of Natural Organic Matter (NOM) in drinking water can readily react with chlorine to form carcinogen compounds and this became a serious problem, but this risk can be reduced by local microbes (*Streptomyces* sp, *Aspergillus* sp, and *Actinomycetes* sp), it utilizes HA as carbon source leads to its decolorization and degradation and this directly showing decrease in aliphatic structural units rapidly than more condensed aromatic structures which were resistant to biodegradation.

CONCLUSION

Different types of organic fertilizer is prepared and followed in various parts of the world but its effectiveness is qualified by humic acid ratio. It is the basic and unavoidable component to qualify any matured organic matter and proper utilization of it to plants. Many researches are carried out for humic acid characterization; UV- spectroscopy is useful and easy tool for preliminary calculation of molecular size of humic acid. Followed by chemical structure analysis by various tech-

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niques is emerging. Application of humic acid in soil instead of direct application of composts will help to direct uptake of nutrients in soluble form by plants. Further investigation on humic acid molecular structure create an awareness on its application in various fields including medicine for its antioxidant properties, water purification, growth supplement in microbial media and also as health drink.

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