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Biosolubilized humic materials with enhanced biological properties

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ABSTRACT
This research is directed to formulation bio-inoculants derived from coal humic substances using biosolubilization technique. Natural microbial population isolated from urbanozem, wood rot of *Ulmis Pamila* and biohumus of vermiculture of *Eisenia foetida* was used as inocula. To characterize the structure of the humic biopreparations obtained, elemental and functional analysis, FTIR, ^{1}H-NMR, ^{13}C-NMR spectroscopy and SEC analysis were used. A seedling technique was used as a test for hormone-like potential of biopreparations.
Keywords: *humic acids, microbial consortia, transformation, hormone-like activity.*

1. INTRODUCTION

Investigations on the mechanisms of degradation of humic compounds are important because of the role of these substances in the global cycling of carbon in the biosphere and their role in plant growth [1]. It is well known that humic substances (HS) can regulate plant growth by interfering with the plant metabolism. Molecular size of HS can be changed by mild chemical reactions, which may take place in the rhizosphere or can be ascribed to soil microorganisms [2].

The findings on auxin-like activity of HS have been supported and extended by experiments showing that some humic fractions have a high hormonal activity [3-5]. In particular, it has been shown that low-weight molecular fraction induced morphological changes similar to those caused by indolacetic acid (IAA) [6]. Moreover, this fraction like IAA increased IAA oxidase activity [7]. In further experiments it has been demonstrated that low-weight molecular fraction of HS binds to the IAA cell membrane receptors [8]. However, there is still no direct chemical evidence that can prove these hormone-like activity activities of HS are due to the presence of plant growth regulators. Concentration of IAA in HS estimated using immunoassays varied from 0.5 to 3.7% (w/w). The results obtained can be related though not only to the presence of IAA, but also to other cross-reacting groups in HS [9]. HS may contain different biologically active compounds – products of biosynthetic activity of microorganisms. For instance, the structure of HS can include polyamines that are plant regulators functioning similarly to the recognized plant hormones. The results of Young and Chen demonstrated that the content of putrescine, spermidine, and spermine in HS of different sources ranged between 1.54-7.00, 0.39-3.88, 0.48-4.79 mM/g, respectively [10]. The authors concluded that polyamines might explain the hormone-like activity of humic substances.

To elucidate transformation of humic substances that is characterized a high biological stability and activity, bioavailability of microbial populations and HS with a high aromaticity should be investigated. Following the goal, the aim of our study was to investigate abilities of natural microbial populations to enhance degradation of HS and plant growth activity. This approach can be considered as a model for supply plants with available nutrients throw the mineralization of organic matter in soils by bacteria and fungi.

2. EXPERIMENTAL SECTION

Humic preparations (HP) used for further biosolubilization were extracted from oxidized coal (Kyzyl-Kyaand Kara-Kechedeposits, Kyrgyzstan, HA1 and HA2) using isolation procedure with 0.1 M NaOH as in ref [11]. The obtained alkaline extract was acidified to pH 1 using concentrated HCl. The precipitated HA were centrifuged (6000 rev/min), washed out with distilled water and dialyzed. HA1 and HA2are 72.75 and 79.54% accordingly.

2.1.Microbiological methods.

Natural microbial population from a cultivated soil (Kyrgyzstan), biohumus from *Eiseniafoetida*, and wood rot from *Ulmis Pamila* were used as inocula. Colony forming units (CFU) were counted after 7 days at 25^{o}C on MPA for bacteria and on Chapek agar for microscopic fungi (standard deviation of CFU counts: 10%). Numbers of bacteria were determined microscopically after fluorescent staining of cells according to Bloem [12]. For the approximate determination of bacterial diversity in samples, 19 bacterial isolates obtained from agar were evaluated using RDP, Genbank and BLASTA.

2.2. Biosolubilization of HS.

The basal solution (per liter of distilled water) KH_2PO_4 – 0.5 g, K_2HPO_4 – 0.5 g, $MgSO_4$ – 0.4 g, NaCI – 0.1 g, $CaCl_2$ – 0.01 g, $(NH_4)_2SO_4$ – 0.5 g, glucose – 15 g, was used in full strength (N) or without $(NH_4)_2SO_4$ to receive a nitrogen-deficient nutrient solution. The salt solution, sterilized by autoclaving (160^{o}C, 30 min), 5 mL of inoculum, and 1 g of HS were added into flasks to final concentration of 1.0 mg/mL. pH was 6.8. The flasks were incubated in the dark at 28^{o}C with shaking for 3, 6, 9, 12 months.

All experiments were performed in duplicate. After incubation biosolubilized HA were separated by centrifugation, washed out with dist. H_2O, desalted using dialysis and dried in vacuum at 60º. Noninoculated sterile HS served as a control. A set of the parent and biomodified samples is described in Table 1.

2.3. Elemental analysis.

Elemental analyses (C, H, N) were performed on a Carlo Erba Strumentazione elemental analyser. Ash contents were determined manually. Oxygen contents were calculated as a difference.

2.4. Determination of total acidity.

5-10 mL aliquot of HA solution containing 5-20 mg HA was transferred into a vial (22 mL) and 10 mL of 0.03 M $Ba(OH)_2$ were added. The vial was tightly sealed, shaken well and left for equilibration for 24 hours at room temperature.

Table 1. List of humic derivatives.

Preparation index	Description
HA1, HA2	Native humic acids Kyzyl-Kiya, Kara-Keche accord.
BHS1-S3, BHS1-S6, BHS1-S9, BHS1-S12	HA incubated with soil microbial populations during 3, 6, 9, 12 months accordingly
BHS1-S3 (Fr1)	Fraction 1 of BHS1-3, 56 kD
BHS1-S3 (Fr2)	Fraction 2 of BHS1-3, 30 kD
BHS1-S3 (Fr3)	Fraction 3 of BHS1-3, 5 kD
BHS1-S12N	BHS1-S12 in N-deficient medium
BHS1-W12	HA1 incubated with wood rot microbial population for 12 months
BHS1-B12	HA1 incubated with biohumus microbial population for 12 months
BHS2-S3, BHS2-S6	HA2incubated with soil microbial populations for 3, 6 months accordingly
BHS2-S3 (Fr3)	BHS2-S3, fraction 5kD
BHS2-S3N	BHS2-S3 in N-deficient medium

Aliquots of transparent solution above the precipitate of Ba humates were transferred to titration cell and titrated with HCl standard solution (~0.1 M) using phenolphtaleine as an indicator. Total acidity (TA, mmol/g) was calculated according to the formula

$$TA = \frac{(V_0 - V_{HA}) \cdot c_{HCl}}{m}$$

where, V_0 and V_{HA} are the volumes of HCl (mL) consumed for blank and sample titrations respectively, C_{HCl} is the titrant concentration (mmol/mL) and m is the mass (g) of HA in the aliquot.

Saturated $Ba(OH)_2$ solution was prepared from BaO by dissolving it in CO_2-free deionized water (boiled during 1 hour) in a sealed volumetric flask under intensive shaking. The solution was left for 3-4 days until complete precipitation of $BaCO_3$ occurred.

2.5. Determination of carboxylic acidity.

5-10 mL aliquot of HA solution containing 5-20 mg HA was transferred into a vial (~ 22 mL) and 10 mL of 0.6 M $Ca(CH_3COO)_2$ were added. The vial was tightly sealed, shaken well and left for equilibration for 24 hours at room temperature.

Aliquots of transparent solution above the precipitate of Cahumates were transferred into titration cell and titrated with NaOH standard solution (~0.05 M) using autotitrator. Carboxyl acidity (CA, mmol/g) was calculated according to the formula

$$CA = \frac{(V_{HA} - V_0) \cdot c_{NaOH}}{m}$$

where, V_0 and V_{HA} are the volumes of NaOH (mL) consumed for blank and sample titrations respectively, C_{NaOH} is the titrant concentration (mmol/mL) and m is the mass (g) of HA in the aliquot.

2.6. UV/vis Spectroscopy.

The light absorbance of HS (200ppm HS redissolved in 0.1 M $NaHCO_3$) was measured at A_{440} nm in pH-M290 (Radiometer).

2.7. Fourier Transform Infra Red Spectroscopy.

Fourier transform infrared (FTIR) spectra were obtained by pressing the HA sample into KBr pellet and analysing with a FTIR spectrometer Magna-750 (Nicolet, USA) on spectral range of 400-4000 cm^{-1}.

2.8. Size Exclusion Chromatography.

Molecular – mass distribution of HS was determined by use of gel-filtration method. Sephadex G-75 and chromatographic columns (20 cm x1cm) were used. Eluation of the fractions were hold by use of Tris-buffer with pH 9.5. The concentration of the introduced in the column HA solution – 5 mg/mL. The optical density was detected by 280 nm.

2.9. Quantitative ^{13}C-NMR Spectroscopy.

13C solution-state NMR spectra of HP samples were measured on solutions of humic materials in 0.3 MNaOD/D_2O at concentration of 100 g/L. Measurements were made on a Bruker AC 400 NMR spectrometer operating at 100MHz 13C observation frequency using inverse gate decoupling at 303 K at aq = 229 msec and aq+d1 = 8 sec (dissolved after extensive ultrasonic treatment; typically dissolved > 70 %, interpulse delay at 90-deg), including section integrals were presented. These NMR spectra represent co-added NMR spectra to improve the S/N ratio, and substantial acquisition time has gone into these data. MeOH/D_2O (d = 49.0 ppm) was used as an external standard. All the spectra were recorded at 8-s delay time. These conditions were shown to provide quantitative determination of carbon distribution among the main structural fragments of HA. To quantify the observed spectra, the assignments were made after Kovalevskii (Kovalevskii, 1998) and were as follows (in ppm): 5-50, aliphatic H and C-substituted C (CAlk); 50-108, aliphatic O-substituted C (CAlk-O); 108-145, aromatic H and C-substituted C (CAr-H,C); 145-165, aromatic O-substituted C-atoms (CAr-O); 165-187, - C of COOH/R groups (CCOO); 187-220 – ketonic/quinoic groups (CC=O).

Two-dimentional NMR spectra were acquired with Bruker DMX 500 NMR spectroscopy operating at 500.13 MHz proton frequency with 5 mm z-gradient cryogenic probe at 303K. The spectra were recorded on 14GK, 5.2 mg of the sample was dissolved in 748.4mg 0,1N NaOD/D_2O. ^{1}H, ^{1}H-correleation

spectroscopy (COSY) spectrum was acquired using standard *cosygpmfqf* pulse program; acquisition time was 0.68 s, relaxation delay 0,82 s. Heteronuclear single quantum coherence (HSQC) spectrum was acquired using standard *hsqcetgpsi* pulse program; F2(^{1}H) acquisition time was 170,5 ms, one-bound coupling constant ^{1}J(CH) = 150 Hz, relaxation delay 1.33 s, number of scans 128; F1(^{13}C): SW=70ppm, 128 increments.

2.10. Quantitative ^{1}H-NMR Spectroscopy.

^{1}H-NMR spectra were acquired with Bruker DMX 500 NMR spectroscopy operating at 500 MHz proton frequency. The spectra were recorded at 303K using the 1-st increment preset-NOESY (90-deg), acquisition time = 4.7 s, relaxation delay = 15 sec.

All the spectra were acquired with 5-mm broadband probe, the samples were dissolved in 700 ml 0.1N NaOD/D_2O.

Bioassay. Auxin-like activity of the HP was assayed by checking the water cress (*Coronopus*) root growth as described in ref. [13]. Gibberellin-like activity of them was evaluated by checking the increase in length of lettuce (*Lactuca Sativa*) hypocotyl. The calibration curves were obtained with IAA and gibberellic acid (GA), supplied by SIGMA. Presented data are means of three replicates. Standard deviations were always within 5%.

3. RESULTS SECTION

3.1. Elemental analysis.

According to the elemental composition of HP recovered from microbial cultures, a decrease in carbon and a significant increase of nitrogen in HPreisolated from the full strength broth inoculated with wood-decay microorganisms has been found (Tables 2, 3).

Table 2. Elemental analysis of the HP.

Sample	Content of elements on ash-free basis, % (mass)					
	C	H	N	O	H/C	O/C
HA1	65.99	3.86	1.08	28.71	0.70	0.36
BHS1-S3	63.30	5.50	0.80	30.35	1.04	0.35
BHS1-S6	64.60	5.80	1.20	28.40	1.07	0.32
BHS1-S9	59.54	5.64	1.99	32.83	1.12	0.41
BHS1-S12	59.50	5.28	1.95	32.83	1.06	0.41
BHS1-S12N	60.97	3.31	1.64	33.31		
BHS1-W3	56.22	4.14	0.97	37.87		
BHS1-W12N	58.92	3.83	2.62	34.06		
BHS1-B12N	61.18	3.13	1.39	33.37		
BHS1-B12	60.73	3.50	0.97	34.24		
HA2	65.51	3.91	1.08	29.22	0.71	0.33
HA2 (Fr3)	62.30	3.80	1.00	32.60	0.73	0.39
BHS2-S3	58.40	4.60	0.80	36.20	0.91	0.56
BHS2-S3 (Fr3)	58.50	4.40	1.20	35.90	0.90	0.50
BHS2-S3N	62.00	5.00	0.90	32.10	0.98	0.40
BHS2-S6	64.20	4.60	1.20	30.00	0.85	0.34

Table 3. Functional group composition analysis of the HP.

Sample	Content of functional groups, mmol/g		
	COOH	Ar-OH	Ar-CHO
HA1	4.21	3.42	1.61
BHS1-S3	5.09	3.62	1.72
BHS1-S6	5.21	4.62	1.80
BHS1-S9	4.42	2.96	1.86
BHS1-S12	3.75	3.12	1.48
HA2	3.84	3.70	1.70
HA2 (Fr3)	3.62	3.50	1.68
BHS2-S3	5.21	4.62	1.54
BHS2-S3 (Fr3)	5.30	4.46	1.61
BHS2-S3N	4.51	3.49	1.41
BHS2-S6	4.74	4.56	1.70

A significant increase of H/C and O/C was also found in the HP. If biohumus microorganisms were used as inoculum, only minor changes were detected in the elemental composition of HP.

3.2. UV/vis Spectroscopy.

In the decoloration tests of the HP natural soil microbial population reduced absorbance (A_{440}) progressively over the test period of 3 months and more (Fig. 1). At the end of month 9, natural microbial populations from soil, biohumus, and wood rot had reduced the absorbance of HS media by 79, 75, and 62%, accordingly.

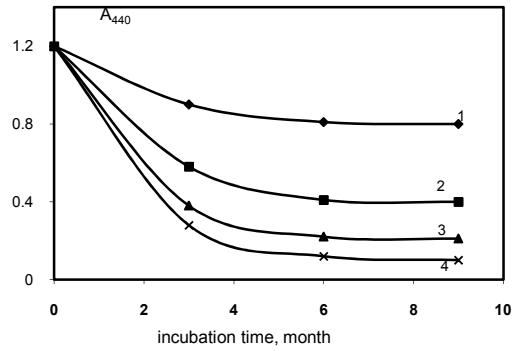

Figure 1. Decoloration of humic derivatives solution by inocula: 1- soil, 2– biohumus, 3– wood rot, 4– no inocula.

3.3. FTIR-spectroscopy.

FTIR spectra of reisolated HS showed (not shown) a decrease in absorption bands at 2362, 1718, 1396, and 1212 cm^{-1} which is due to the disappearance of C-C and C-O bonds in various structural units (ketones, alcohols, phenols, carbohydrates). Differences in the bands at 1525-1565 cm^{-1} can be attributed to alteration of proteinaceous structures. The FTIR spectra of HS reisolated from full broth media inoculated with soil microorganisms showed strong absorbance at 1663 cm^{-1} (amide I), 1034 cm-1 (polysaccharides), and 2926 cm^{-1} (aliphatics).

3.4. Molecular weight changes of the HP.

Changes in concentration and molecular wt composition of HS fraction incorporated into culture medium, which had been monitored at regular times during the incubation at 280 nm, are shown in Table 4. A relative reduction of the molecular weights was noticed after 3 months incubation, and accumulation of new low molecular weight fraction after 6 months incubation was recorded after chromatography on Toyopearl HW-50S. Reductions in amount were due to a random degradation of substances in all

molecular size classes. A formation the high molecular wt fraction has been found.

Table 4. Molecular-mass distribution of the HP (kD/%).

Fraction	Fr-1	Fr-2	Fr-3	Fr-4
HA1	60/46.2	40/30.8	15/23.0	-
BHSS1-3	56/15.2	30/53.4	5/31.4	-
BHSS1-6	>80/21.6	45/30.6	12/29.4	5.0/18.4
HA2	50/40.4	32/36.1	15/23.5	-
BHSS2	49/14.8	30/40.4	5/44.8	-
BHSS2-N	50/25.3	30/49.8	5/24.9	-
BHSS2	>80/25.0	42/38.0	15/20.5	5.0/16.5

^{13}C, ^{1}H -NMR-*spectroscopy*. The data obtained by ^{13}C-NMR spectra of the investigated humic substances indicated the spectra are typical for those of coal humic acids, characterized by large peaks corresponding to aromatic (100-165 ppm) and carboxylic/ester (165-187 ppm) signals and reduced hydrocarbon (48-108 ppm) signals. Aromatic carbon signals increase as a result of microbiological long treatment of the HA. At the same time, aromatic hydrogen signals decrease from the HA to the microbiological treated HA. It can be interpreted as a result of carbon substituted aromatic rings contribution relatively increased. The ^{13}C-NMR-spectra of hematomelanic acids, in comparison with the HAs, demonstrated increasing in the intensities of the hydrophobic (alkyl and non-substituted by heteroatoms aromatic) groups signals. The table also shows increasing of aromatic spectral signals intensity (108-145 ppm) and decreasing of spectral integrals in the 58-90 ppm area, corresponding to hydrocarbon and peptide functional groups of studied HP, as a result of the HP long time treatment by microorganisms.

Complementary information provided by the homonuclear $^{1}H,^{1}H$-COSY spectrum acquired is accordant to the heteronuclear spectroscopy the data obtained (Table 5).

Table 5. ^{1}H-NMR spectral integrals of the HP (%).

Structural fragments:	Aromatic H	O-CH-O,N	CH-O,N	alpha-CH*	Alkyl-H
Intervals, ppm:	10.0-6.0	6.0-4.8	4.6-3.2	3.2-2.05	2.05-0.0
HA1	52.7	1.8	2.7	8.2	33.6
HA2	51.4	3.1	1.8	8.2	33.6
BHS1W12	49.6	0.9	4.5	9.9	33.3
BHS1W12-N	55.9	0.0	0.9	9.9	32.4
BHS1S12	53.6	1.8	3.6	9.8	29.5
BHS1S12-N	48.2	1.8	2.2	8.9	37.5
BHS1B12	49.6	0.9	3.6	9.9	33.3
BHS1B12-N	45.9	0.0	1.8	9.0	42.3

* *alpha-CH* – protons of aliphatic groups in alpha-position to electronegative group or to aromatic ring

The $^{1}H,^{1}H$-COSY spectrum can be divided into two areas of chemical shifts: (1) long-chain aliphatic groups (0.6-1.5ppm) and (2) pure aliphatics bounded with functional aliphatics (1.5-2.4ppm). The signal at 2.3-2.4 ppm seems to correspond with alkylated nitrogen; the cross peak at 1.4-1.5/2.3-2.4ppm in the COSY-spectrum shows that these structural fragments are bounded with pure alkylated groups.

Comparison of the ^{1}H and ^{13}C NMR spectroscopy results shows the following: aromatic carbon signals increase as a result of microbiological long treatment of the HAs. At the same time, aromatic hydrogen signals decrease from the HAs to the microbiological treated HAs. It can be interpreted as a result of carbon substituted aromatic rings contribution relatively increased. The significant rise of alpha-groups proton signals shown in the Table 5 confirms this supposition.

Table 6. Prominent cross signals in the HSQC spectrum of BHS1-B12.

δ (^{13}C), ppm	δ (^{1}H), ppm	Tentative assignment
12-15	0.6-0.7	CH_3-terminal groups in a long aliphatic chain
20-24	1.0-1.1	CH_2 bounded with terminal methyl groups in a long aliphatic chain
26-31	0.9-1.15	CH_2 groups in a long aliphatic chain
27-29	1.4-1.5	different pure alkyl groups
33-37	1.4-1.5	
32-38	1.7-1.85	
31-34	2.0-2.2	alkyl groups in α-position to functional groups, or to aromatic rings, or in ß-position to OH-functions
53-57	2.3-2.4	nitrogen-substituted alkyls

3.5. Two-dimentional NMR spectra - ^{13}C, ^{1}H-HSQC spectrum.

Detail two-dimentional NMR spectroscopic study of aliphatic part of BHS1-B12 (Table 6) shows that this substance is characterized with long alkyl chains and nitrogen-substituted aliphatic groups. Any sugar and polysaccharide fragments are absent.

3.6. Biotesting.

The selectivity of impact of HS different fractions on the plant growth has been observed (Table 7).

Table 7. Auxin- and gibberellin-like activity of the HP.

Sample	Mw, kD	Preparation levels (mg C/L) having hormone-like activity corresponding to 1 mg/L of IAA	to 1 mg/L of GA
HA1	44	–	–
HA1 (Fr2)	40	–	$3.3 \cdot 10^{-1}$
HA1 (Fr3)	15	$4.1 \cdot 10^{-1}$	-
BHS1S3 (Fr2)	30	$8.4 \cdot 10^{-3}$	$2.4 \cdot 10^{-3}$
BHS1S3 (Fr3)	5	$2.1 \cdot 10^{-2}$	$3.0 \cdot 10^{-2}$
BHS1S6(Fr-3)	12	$2.8 \odot 10^{-2}$	$3.2 \odot 10^{-2}$
BHS1S6(Fr-4)	5	$3.5 \odot 10^{-2}$	-
BHS1S12	15	$7.8 \cdot 10^{-3}$	$8.0 \cdot 10^{-3}$
HA2	35	–	–
HA2 (Fr-2)	38	-	$6.2 \cdot 10^{-2}$
HA2 (Fr-3)	15	$1.2 \cdot 10^{-3}$	–
BHS2S3 (Fr1)	56	-	-
BHS2S3 (Fr2)	40	-	$3.4 \cdot 10^{-2}$
BHS2S3 (Fr3)	5	$6.4 \cdot 10^{-2}$	$3.0 \cdot 10^{-2}$
BHS2S6 (Fr1)	42	-	$3.2 \cdot 10^{-3}$
BHS2S6 (Fr2)	15	$7.9 \cdot 10^{-3}$	$8.7 \cdot 10^{-3}$
BHS2S6 (Fr3)	5	$5,8 \cdot 10^{-2}$	-

Parent HA1 and HA2 do not have hormone-like activity. A hormone-like activity has been showed by HP preparations which were characterized with low molecular weights. Each of these preparations was endowed with a single specific (auxin-like or gibberellin-like) activity. Biosolubilized HP with low molecular weight displayed two kinds of activity.

4. CONCLUSIONS

Coal humic acids (HA) from oxidized brown coal (Kara-Keche and Kyzyl-Kiya Kyrgyz deposits) were isolated and added to a Czapek nutrient broth which was used either in full strength or without $(NH_4)_2SO_4$. The individual flasks were inoculated with natural microbial populations of corresponding cultivated soil, biohumus and wood rot samples for 12 months.

According to the elemental composition of HP recovered from microbial cultures, a decrease in carbon and a significant increase of nitrogen in HS reisolated from the full strength broth inoculated with wood-decay microorganisms has been found. If biohumus microorganisms were used as inoculum, only minor changes were detected in the elemental composition of HS. A significant increase of H/C and O/C was also found in the HS. It can be attributed to formation new aliphatic and O-containing structures and decrease aromatic ones. Accumulation of fulvic acids was recorded after 6 months incubation. At the end of month 9, natural microbial populations from soil, biohumus, and wood rot had reduced the absorbance of the HP media by 79, 75, and 62%, respectively.

A relative reduction of the molecular weight was noticed after 3 months incubation, and accumulation of new low molecular weight fraction after 6 months incubation was recorded after chromatography on Toyopearl HW-50S. Reductions in amount were due to a random degradation of substances in all molecular size classes. A formation the high molecular weight fraction has been found. It can be caused by cross-linking of structural constituents of molecules due to radicals forming after biodestruction or by their interaction with metabolites.

The data obtained by spectroscopic methods (UV/vis/FTIR, 13C NMR) and element analysis indicated a decrease in particle size and a loss in aromaticity and aliphatic carbon in HS reisolated from microbial cultures. Simultaneously an increase in the N content of HS was observed, which probably from some constituents of microbial biomass such as proteins and amino sugars. The microbial degradation of HS strongly depended on the composition of the HS, the species selection of the microorganisms, and to a lesser extent on the culture conditions.

A hormone-like activity has been showed by HS preparations which were characterized with low molecular weights (~5-15 kD). Each of these preparations was endowed with a single specific (auxin-like or gibberellin-like) activity. Biosolubilized HP with low molecular weight were displayed two kinds of activity. Growth stimulating activity of humic substances biosolubilized up to substances with small molecular weight is comparable with phytohormonal activity. This fact can clarify the question how substances with high molecular weight may affect plant metabolism.

Thus, using aimed biochemical modification of humic acids structure it is possible to increase significantly their biological activity, namely plant growth promoting potential. That is very important for the production of humic growth regulators of plants.

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