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Plant Growth Biostimulants from By-Products of Anaerobic Digestion of Organic Substances

Sharipa Jorobekova and Kamila Kydralieva

Abstract

The by-products of anaerobic fermentation of agricultural wastes as a result of methanogenic microorganism activity are various bioactive substances, including humic-like substances (HLS). The contents of HLS formed are changed during fermentation process. The degree of humification significantly ranges on different fermentation stages reflecting the biosynthetic activity of microbial consortium in the processes of maturing and transformation of humic compounds. Characteristics of HLS isolated on various fermentation stages and bioactivity assessment present much interest for future applications as plant growth biostimulants, organic-mineral fertilizers, and phytohormones.

Keywords: humic-like substances, anaerobic fermentation, biostimulants, phytohormones

1. Introduction

Anaerobic digestion of organic wastes including manure with other substrates such as energy crops, industrial wastes, or food industry wastes is a commonly-used method as it can transform organic matter into biogas [1, 2]. During waste anaerobic digestion, the degradation of organic substances is commonly divided into the following stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis [3–6]. High molecular weight (MW) compounds, such as lipids, polysaccharides, proteins, and nucleic acids, are degraded into soluble organic substances (e.g., amino acids and fatty acids) and then split further into volatile fatty acids, ammonia (NH_3), CO_2 , H_2S , and other by-products. The higher organic acids and alcohols are further digested to form mainly acetic acid, CO_2 , and H_2 , which are used to produce methane by different methanogens [7]. Besides proteins, polysaccharides, lipids, and nucleic acids, humic-like substances (HLS) are also major organic constituents of liquid digestate [3], sludge [8, 9], and their contents were reported to reach 26–28% of sludge organic matter [10, 11].

There are two major theories regarding HSs formation. Firstly, in the lignin theory, HSs are synthesized from precursors originating from lignin, meaning that lignin is the raw material and skeleton of HS precursors [12]. According to Kulikowska [13], the partial degradation of lignin can form phenolic and quinone moieties that can serve as HS precursors. Secondly, in the polyphenol theory, HSs are the condensation products of many small molecules, such as polysaccharides and proteins [12].

When compared with commercial humic substances, digester and sludge humic substances contain a wider variety of organic substances, more lipids, more nitrogen, and a lesser degree of oxidation. However, there are no significant differences in the effects of the two types of humic substances on plant growth [14]. Consequently, humic acids and fulvic acids could be extracted from digested sludge as a new source of organic liquid fertilizer, i.e., biostimulants. According to the definition by Jardin [15], plant biostimulant is any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance, and/or crop quality traits, regardless of its nutrient content. Application of humic substances—soluble humic and fulvic acid fractions—shows inconsistent, yet globally positive, results on plant growth. A recent random effect meta-analysis of HS applied to plants [16] concluded on an overall dry weight increase of $22 \pm 4\%$ for shoots and of $21 \pm 6\%$ for roots. The variabilities in effects of HS are due to the source of the HS, the environmental conditions, the receiving plant, and the dose and manner of HS application [16].

2. Effects of anaerobic digestion on humic-like substance values

2.1 Extraction of humic-like substances

In order to extract humic acids and fulvic acids, some disintegration methods are applied to disrupt flocs and cells and release inner organic substances. The disintegration methods include mechanical, thermal, chemical, ultrasonic, and biological treatments [17]. Some methods can also be combined to disintegrate sludge [18–23]. As one of these methods, alkaline treatment has the advantages of simple devices, easy operation, and high efficiency. Alkaline sludge pretreatment was reported to enhance the dissolution of organic substances [24, 25] and also make humic substances be released from sludge particles [26]. Whether for sludge disintegration or for the extraction of humic substances, sodium hydroxide (NaOH) was more efficient than calcium hydroxide [$\text{Ca}(\text{OH})_2$] [26]. After humic substances are transferred from waste solid phase into liquid phase by alkaline treatment, the dissolved humic substances in the supernatant can be recovered by ultrafiltration separation [2].

2.2 Evolution of humic-like substances during anaerobic sludge digestion

During waste anaerobic digestion, the humic substances evolved compared with that of other organic substances, i.e., proteins, polysaccharides, lipids, and nucleic acids. According to their solubilization in acidic or alkaline solution, HS can be divided into humic acids (HAs), fulvic acids (FAs), and humin (HU). HSs are generally recognized as being nondegradable or hard degradable during wastewater treatment processes, and the removal of HS from wastewater is attributed to biosorption of activated sludge instead of biodegradation [27]. However, HS may be generated by microbial activities during waste storage and treatment [28]. Sludge was found to be enriched in oxygen functional groups and aromatic rings during the course of storage and composting [29–31], but the humification process is not complete due to the typically low free radical concentrations and the unstable C/N ratio [32]. On the other hand, HSs were also found to be utilized by microorganisms as a supplementary source of nutrients [33]. HAs increased at first and then decreased in landfill composed of municipal refuse and sludge because both humification and mineralization processes took place [33, 34]. Most researches on HS evolution have been confined to sludge composting, landfilling,

and storage. Few studies have investigated that HS might be dynamically involved in carbon and electron flow in anaerobic environments [35, 36]. This electron transfer would yield energy to support growth and stimulate the mineralization of organic compounds under anaerobic conditions [37, 38]. Bartoszek et al. [30] found that HAs became enriched in oxygen functional groups and aromatic rings in a digestion chamber.

During sludge anaerobic digestion, 16.3% of HAs and 27.0% of FAs were degraded, but the degradation rate was relatively low compared with that of other organic substances in sludge. Besides the mineralization of sludge HS, humification processes also took place. The HS extracted from the digested sludge have more oxygen functional groups, more aromatic structures, and larger molecular sizes compared with the HS extracted from the raw sludge. However, the degree of humification was low, and mineralization was still the main process that occurred during sludge anaerobic digestion [2].

2.3 Dynamics of physico-chemical parameters: case study

Fermentation process is carried out at the disposable or periodic loading of the bioreactor by common raw materials of fermentation such as animal manure, sewage sludge, food wastes, and green wastes (the optimal C/N ratio is 20–25). Animal manure and sewage sludge are characterized by a high moisture content, a low C/N ratio, and a low porosity [39, 40]. Green wastes have a low moisture content as well as a high C/N ratio, porosity, and lignification degree [41]. Food wastes are characterized by high levels of salt, grease, carbohydrates, and moisture [42, 43]. Because of the different elemental compositions and properties of raw materials, HAs have different characteristics [44].

Concentration of solids in fermentation medium at the disposable loading of the bioreactor has been increased and data correlate with the increase of amine nitrogen content that verifies biosynthetic processes (Table 1). According to dynamics of enzymatic activities, proteolytic activity of microorganism population decreases by 30 days and the increase of amine nitrogen could not be connected directly with the processes of proteolysis and obviously is the reflection of biosynthetic activity of microbial population. Data on the number of reducing sugars (their number reduction by 30–70 days) are logically kept within the assumption about the change of carbon source (transfer from utilization of easily hydrolyzed substrates to the using of hardly hydrolyzed) and, obviously at this stage of fermentation, the processes of cellulose hydrolysis have been intensified, that is, confirmed by the increase of cellulolytic activity of microbial population. On the basis of data obtained (humic

Sample	pH	D ₄₅₀	Amine N (mg/ml)	Reducing agents (%)	HLS (%)	E ₄ /E ₆ *
PAF20	7.9	0.38	0.63	0.35	1.4	4.1
PAF30	7.6	0.52	0.89	0.33	1.5	7.1
PAF70	7.9	0.68	0.56	0.18	7.6	6.9
PAF100	8.1	1.35	1.12	0.34	8.4	6.8
PAF150	7.1	1.55	1.82	0.39	10.2	3.8
PAF200	6.8	3.50	2.73	0.50	13.1	11.0

*The E₄/E₆ ratio is considered to be inversely related to the degree of aromaticity of the humic substances and to their degree of humification

Table 1.
 Characteristics of PAF samples on different fermentation stages.

substance yield), it can be concluded preliminary that from 70-th day of fermentation, the stage of humic substances' synthesis starts.

Content of HLS formed in the process of fermentation has been increasing to the end of fermentation and partially correlated with the increase of solids, amine nitrogen, and reducing substances. However, the degree of humification significantly ranges on different stages, probably showing biosynthetic activity of microbial consortium on the accomplishment of the processes of “maturing” and “transformation” of humic compounds. The inclusion of the decomposition products of easily degradable compounds, i.e. sources of carbon and the products of cellulose hydrolysis determines the degree of humification corresponding to soil humic acids which contain significant amount of aliphatic fragments, carbohydrates, peptides, and small proteins. It is logical to assume that the reduction of this parameter from 7.1 to 6.8 reflects larger content of humic acids in the preparations as compared to fulvic acids, i.e., testifies about the increase of aromatic structures and the degree of condense. Sharp decrease of this parameter to 3.8 indicates about the production of highly condensed compound with high content of aromatics—actually about the formation of humic compound nucleus. From the other side, the presence of enzymatic activities, proteolytic, hydrolase, and cellulase in microbial population on these terms of fermentation, allows to assume the disintegration of aliphatic fragments, carbohydrates, and peptides that significantly reduce the determining value E_4/E_6 . Increase of E_4/E_6 on the last stages of fermentation indicates the appearance of new synthesized macromolecules.

Preparations of HLS extracted from PAFs had a good solubility in low alkaline solutions at pH 12 and precipitated well from the solution at their acidification to pH 2; i.e., HLS demonstrate the properties of natural humic acids (HA) close to the class of microbial and soil humic substances in terms of element composition and spectral characteristics (**Table 2**; **Figures 1** and **2**). Absorption spectra of alkaline solutions of the produced preparations in UV and visible field of spectra presented descending curves without specific strips of absorption (**Figure 1**). Significant absorption in UV field, reducing with the increase of wave length and small “shoulder” at wavelength 280 nm was specific for the studying preparations as well as for natural humic acids.

FTIR spectra allowed not only to assess qualitative composition of the functional groups but also to propose the model of their synthesis and transformation in the process of anaerobic fermentation. A wide intensive band of absorption at $3152\text{--}3270\text{ cm}^{-1}$ corresponds to valent oscillations of OH group (**Figure 2**). Small peaks in the field $2923\text{--}2924\text{ cm}^{-1}$ could be caused by the oscillations of the aliphatic groups CH_2 and CH_3 . There is a small peak in the field 2587 cm^{-1} on the spectrum of PAF-150, obviously responsible for OH-group oscillations. Greatly expressed peak of absorption with maximum at 1667 cm^{-1} on PAF spectrum is a characteristic

Sample	Atom % in HLS						
	C	H	N	O	H/C	O/C	C/N
HLS PAF20	41.2	34.8	4.1	19.9	0.84	0.48	10.0
HLS PAF30	34.4	39.3	4.0	22.1	1.14	0.64	8.6
HLS PAF70	34.3	38.1	4.3	23.2	1.11	0.68	7.98
HLS PAF100	35.5	38.4	3.6	22.6	1.08	0.64	9.87
HLS PAF150	41.8	30.5	4.1	23.6	0.73	0.56	10.45
HLS PAF200	33.9	41.5	3.8	20.8	1.22	0.61	8.92

Table 2.
Elemental analysis of HLS.

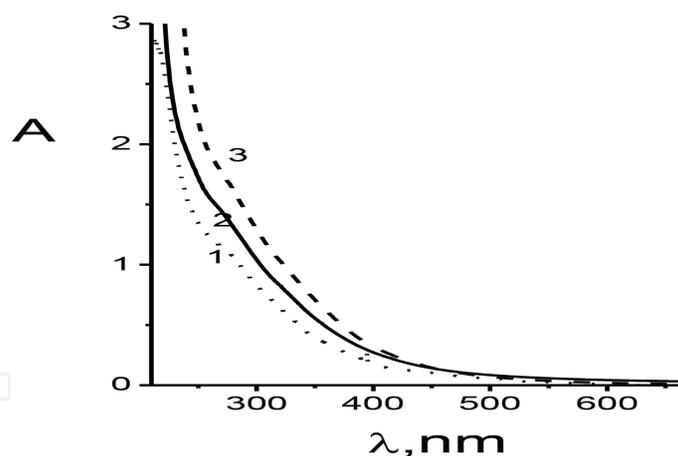


Figure 1.
Electron spectra of HLS: (1) soil HA; (2) HLS PAF₁₅₀; (3) HLS PAF₂₀₀.

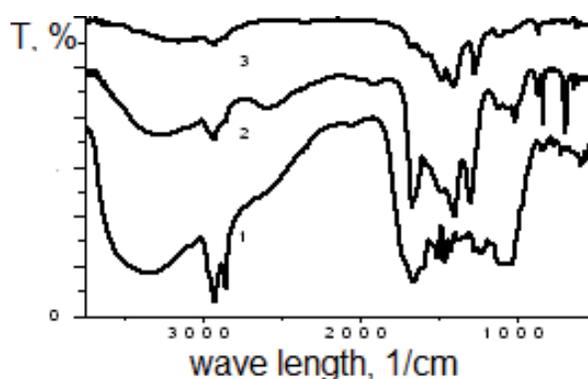


Figure 2.
FTIR spectra of HLS: (1) soil HA; (2) HLS PAF₁₅₀; (3) HLS PAF₂₀₀.

for double bonds C=C, —CH=CH₂, and =C=CH₂. For PAF 200 spectrum, the absorption in this field is expressed weaker. On both spectra, there is a peak at 1570–1577, responsible for the oscillation of C=N bond, and also of aromatic structures. The peak near 1470–1473 cm⁻¹, responsible for the oscillations of CH₂ and CH₃ groups, is distinctly presented on PAF 200 spectrum, to more extent than on PAF-150 spectrum. Distinct peak on both spectra in the field of 1396–1406 cm⁻¹ can be attributed to the oscillations of COOH and —CHO groups. Absorption in this field is greatly expressed on both spectra. Primary and secondary alcohol groups could be responsible for the peak in the field 1270–1298 cm⁻¹. Absorption on both spectra in the field 1131 cm⁻¹ can be linked with the oscillations of CO-alcohol and carbon groups. The availability of the above-mentioned atomic groups is an indication that the isolated preparations of humic substances are close to the other microbial humic-like substances and also to natural humic acids.

HLS of PAF presented two fractions according to size-exclusion chromatography analysis. One fraction goes out by sharp peak into the field of free volume of the column (it is also the characteristic for soil humic substances). It indicates that in all studied preparations there is high molecular fraction by molecular mass about 80 kD (determined from calibration curve). Low molecular fraction corresponds to about 5 kD by molecular wt. This peak was also sharp by form and differed from the wide peak on elution curve of soil humic substances with molecular weight about 23 kD.

Electropherograms of capillary electrophoresis typical for the HS from PAF samples (data are not shown) are similar to the electropherograms of the HA standards. Compounds migrating in 10–15 min interval and presented in the electropherograms as peaks with uneven front and extended end (“hump”) are presented.

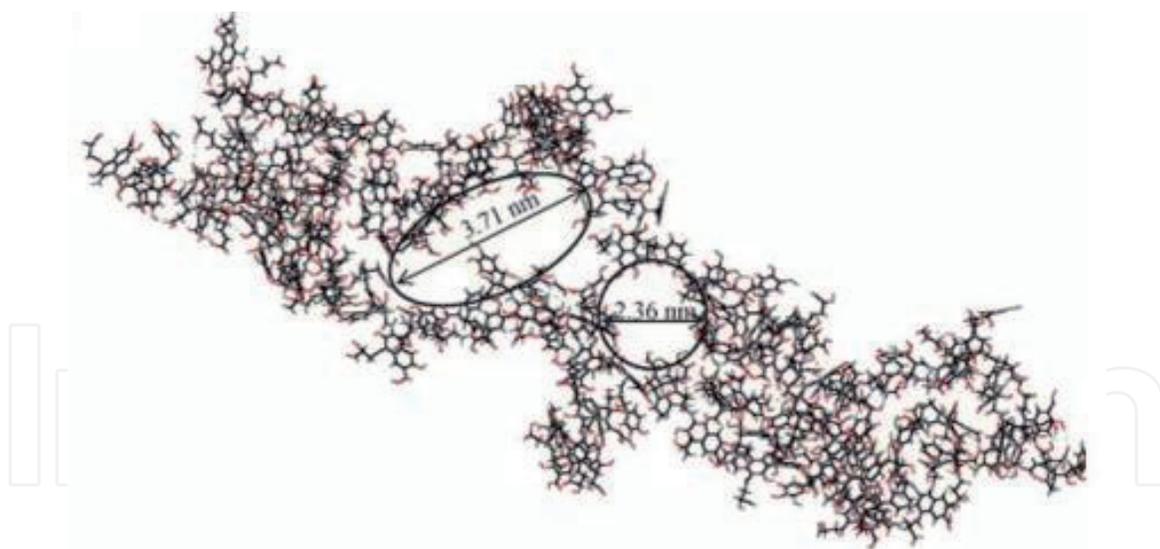


Figure 3.
Formula of humic substances [45].

The amount of the HS formed during a fermentation increases up to the end of a fermentation. The humification degree sharply changes at various stages.

According to Alvarez-Puebla et al.'s HS model [45], simple (though heterogeneous) monomeric units progressively build up into high-molecular weight polymers by random condensation and oxidation process (**Figure 3**). Accepting this model, we can assume the following mechanism of humic substance formation in the process of anaerobic fermentation: the first stage includes microbiological synthesis of humic substance nucleus, containing a great number of aliphatic fragments (initial period of fermentation). Next inclusion into synthesized nucleus of aromatic structures and/or the transformation of aliphatic fragments into aromatic structures takes place in the period between 30 and 150 days. The humic substances produced by their properties, spectral and spectroscopic characteristics, are mostly close to natural humic substances. The second stage includes further transformation of humic substances (150–200 days of fermentation) and can represent two processes: inclusion and/or formation of aromatic structures and hydrolysis of humic substances by microorganisms. As shown in [35], humic substances can play a role of electron acceptors for anaerobic respiration of microorganisms, as redox mediators for the processes of recovery and as donors of electrons for microorganisms. It should be noted that *Bacillus subtilis* can use quinone derivatives as donors of electrons and take part in the fermentation process and can participate in the transformation of humic substances. The third stage actually confirms the completion of high aromatic structure formed in the second stage and formation of aggregates. The stability of HS aggregates in solution is dynamic and influenced by solution ionic strength and pH.

2.4 Plant growth enhancement

Humic substances contribute to the growth and health of agricultural plants [46]. Moreover, HAs have been reported to have positive effects on the growth of wheats, ornamental plants, peas, and many other economically valued plants [47–49]. A recent random-effect meta-analysis of HS applied to plants [16] concluded on an overall dry weight increase of $22 \pm 4\%$ for shoots and of $21 \pm 6\%$ for roots. HSs can promote plant growth, which seems to be related to their positive influence on root architecture and the soil environment. Nardi et al. [50] revealed that HAs can promote the uptake of Na, Ba, and P in plants and modify the pH of the soil surrounding the root by stimulating the activity of H^+ -ATPase in plant roots. Sharif et al. [51]

revealed that application of HAs to potted corn significantly increased root and shoot biomass, while Tahir et al. [47] found that the largest increase in plant height and shoot weight occurred when HAs were at a concentration of 60 mg/kg soil. In addition, HAs increase the cell membrane permeability, which can increase nutrient uptake and accumulation [47, 49]. In summary, the application of HSs can enhance the seed germination, rooting, seedling growth, and nutrient use of plants. HSs are therefore ideal for use in place of synthetic plant growth regulators.

According to a review by Jardin [15], humic substances have been recognized for long as essential contributors to soil fertility, acting on physical, physico-chemical, chemical, and biological properties of the soil. Most biostimulant effects of HS refer to the amelioration of root nutrition, via different mechanisms. One of them is the increased uptake of macro- and micronutrients, due to the increased cation exchange capacity of the soil containing the polyanionic HS, and due to the increased availability of phosphorus by HS interfering with calcium phosphate precipitation. Another important contribution of HS to root nutrition is the stimulation of plasma membrane H^+ -ATPases, which convert the free energy released by ATP hydrolysis into a trans-membrane electrochemical potential used for importing nitrate and other nutrients. Besides nutrient uptake, proton pumping by plasma membrane ATPases also contributes to cell wall loosening, cell enlargement, and organ growth [52]. HSs seem to enhance respiration and invertase activities providing C substrates. The proposed biostimulation activity of HS also refers to stress protection. Phenylpropanoid metabolism is central to the production of phenolic compounds, involved in secondary metabolism and in a wide range of stress responses. High-molecular mass HSs have been shown to enhance the activity of key enzymes of this metabolism in hydroponically-grown maize seedlings, suggesting stress response modulation by HS [53, 54].

It has been stated that digestates contain bioactive substances, such as phytohormones (e.g., gibberellins and indoleacetic acid), nucleic acids, monosaccharides, free amino acids, vitamins and fulvic acid, etc., with the potential to promote plant growth and to increase the tolerance to biotic and abiotic stress [55]. Digestates have higher contents of indoleacetic acid than the original plant feedstock [56]. This increase could only be explained by a microbial synthesis during the digestion process. The biotests of the identified fractions of HLS showed that all the fractions are active. But the efficiency of stimulating assay depends on the

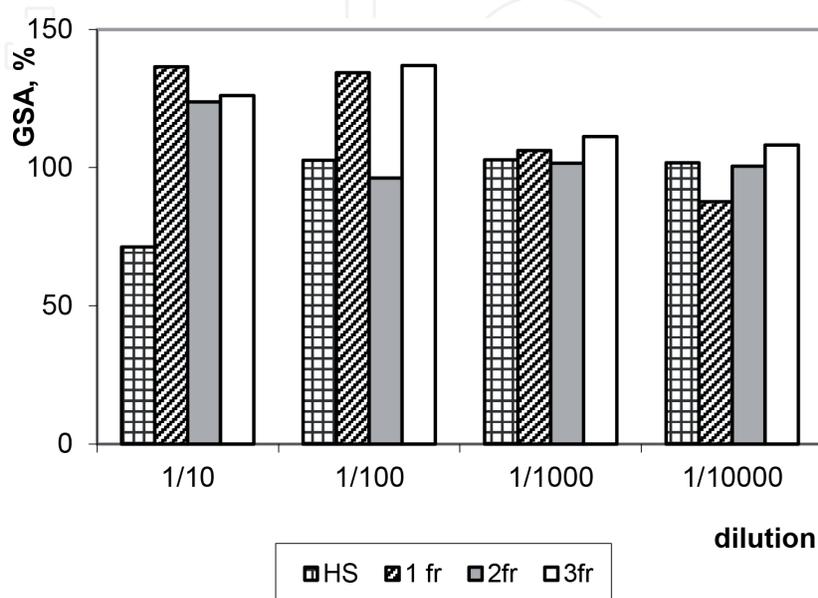


Figure 4. Influence of HLS on the wheat seed germination: (1) PAF₇₀; (2) PAF₁₀₀; (3) PAF₁₅₀ (GSA—growth-stimulating activity, 1fr—fraction with molecular weight ca. 5 kD, 2fr—ca. 50 kD, 3fr—ca. 100 kD).

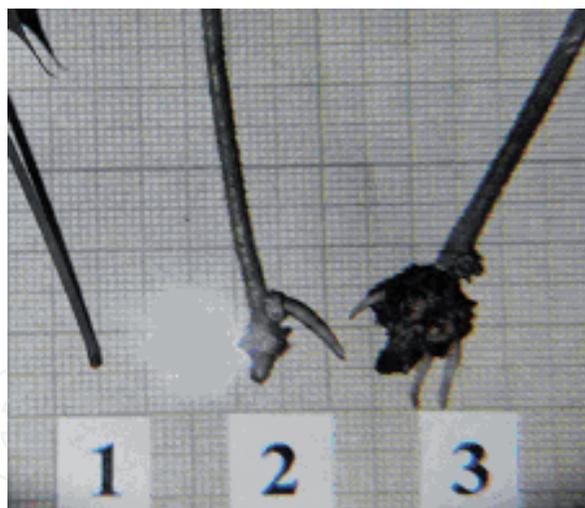


Figure 5. Auxin biotest: (1) control; (2) 10^{-6} M IAA; (3) 0.1 g/L HLS PAF70 (the auxin-like activity of HLS was estimated using the tillers of liana sp. *Cissus L.* of Vitaceae. The callus produced and root system of tillers was used as an auxin-test response. Tillers of liana were placed in water solution (1), in 10^{-6} M of IAA (indole-acetic acid) (2), and in 0.1 g/L of HLS of PAF70 (3). The rhizogenesis was observed for 60 days at 25°C).

dilution degree. In our case, the specific biological activity of the separated fractions of HS has been determined (**Figure 4**). The effect of “mutual exclusion” of the specific biological activity has been established in case of the unfractionated HP.

The biological system test on the rhizogenesis of liana *Cissus L.* with a high IAA-oxidase activity toward the exogenous auxins has shown an auxin-like effect of HA (**Figure 5**). The size of callus in the sample with HLS significantly increases such as in IAA sample. It can be assumed that there are auxins in the concentration 10^{-6} – 10^{-7} M for HLS. Although hormonal effects are described, whether HSs contain functional groups recognized by the reception/signaling complexes of plant hormonal pathways, liberate entrapped hormonal compounds, or stimulate hormone-producing microorganisms is often unclear [57].

The stimulating effect of HP samples depended on metal ions, and in the field of low concentrations for any of the metal ions, the effect was higher than of model solution of metal ions (**Figure 6**). The increase of metal concentration leads to the increase of growth-stimulating activity of model solution. The increase of metal ions concentration has no clear stimulating effect of humic preparations. The HP inhibit the roots growth at the 10 times dilution.

The inhibiting effect of HLS of PAF to the germination of roots is identical to inhibition effect of the salted soils that may be caused by a high content of osmotic components (OC). The concentration of sodium ions in HLS of PAF-70-200 was 10–24 g/L, and in PAF-20 and PAF-30, it exceeded the limited value. Concentrations of K ions in all the samples also exceeded the detection limit. To differentiate the effect of metal ions and HA the GSA was compared for the following model solutions: (1) metal ions, (2) metal ions with HA, and (3) metal ions with HA and osmotic components (Na^+ and K^+). The effect of osmotic components on growth-stimulating activity is illustrated in **Figure 7**.

In all series of the experiment, a dose-dependent effect of growth-stimulating activity on the concentration of the components of model solutions and HLS of PAF-150 is observed. Comparative analysis of the curves shows that without OC the growth-stimulating activity of model solutions increased with the metal concentration and the presence of HS increased the the roots growth as compared to the metal solution. Addition of the OC to the medium for seed soaking caused the reduction of growth stimulation, even to the inhibition of seed germination.

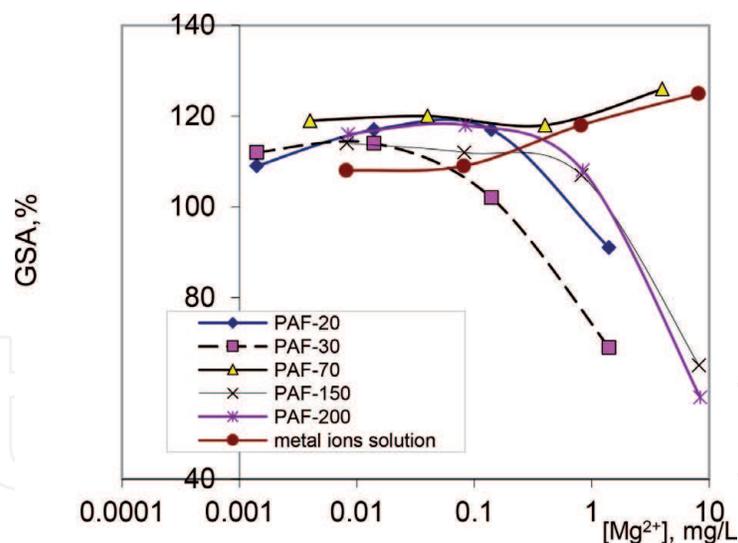


Figure 6. Effect of Mg^{2+} on HLS growth-stimulating activity (Comparative biotesting. Seedling technique was used. Seeds of wheat Lada were incubated for 3 days at $24 \pm 1^\circ C$ at constant illumination. As a control, HLS PAF-150 was used. Concentration of HLS was selected so as the optic density value of model solution corresponded to that in HLS PAF-150. pH of model solution of metal ions is 5.5. The length of roots was used as a test response).

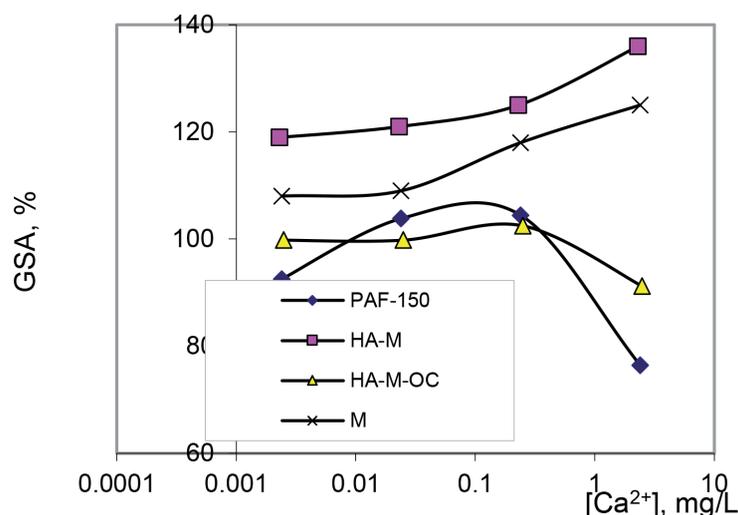


Figure 7. Effect of Ca^{2+} on growth-stimulating activity of PAF-150 and HA (PAF₁₅₀—products of anaerobic fermentation in 150 days, HA-M—humic acids with Ca^{2+} , HA-M-OC—humic acids with Ca^{2+} and osmotic components, M— Ca^{2+}).

2.5 Membrane filtration of the active fractions of humic preparations

The comparison of micro- and ultrafiltration processes allows to produce the preparations with different level of physiological activity (growth-stimulating activity, GSA). The process of microfiltration allows to produce in the permeate the final fraction that stimulates the growth of the roots approximately for 60%. The observing dynamics of an increase in growth-stimulating activity in the permeates allows to make a conclusion about the degradation of humic complex at the reduction of ionic strength of the solution and/or the removal of metals from the complexes of metal-humic substances. As a result of such degradation, we observe the appearance of the fractions with higher physiological activity, and this tendency is a characteristic not only for the permeates but also for the concentrates. The developed technological approach gives a possibility to separate the solid phase from

the target product of the fermentation not only for one stage but also to increase significantly its physiological activity. Besides, this approach can be recommended for the production of low molecular fractions of humic substances of different origin, which are rather prospective from one side, for the study of their effect to membrane transport in plant and microbial cells, and from the other side, it could be used as active additives to the applied organic-mineral fertilizers.

3. Conclusion

The basic biological active components of liquid anaerobic fermentation by-products are the substances of the humic-like nature. Although humic substances may be useless for methane production during anaerobic digestion, they are useful raw materials for organic fertilizers. Common commercial humic fertilizer is primarily produced from peat, brown coal, and weathered coal. When compared with commercial humic substances, sludge humic substances contain a wider variety of organic substances, more lipids, more nitrogen, and a lesser degree of oxidation [2]. The process of anaerobic fermentation of the mixed wastes of plant and animal origin reflects the dynamics of microbial population change and humic-like by-product evolution. The content and composition of humic-like substances on different stages of fermentation are varied. By behavior in alkali, acids, element composition, spectroscopic characteristics, data of capillary electrophoresis, and size-exclusion chromatography, the humic-like substances as by-products of organic waste anaerobiosis are close to the class of microbial and soil humic substances.

The level of growth-stimulating activity of humic-like substances depended on metal content in the products of anaerobic fermentation. The process of microfiltration to extract bioactive fractions could be used for the isolation of two types of fractions: one of them is the fractions close to natural humic substances, and the second is plant hormone-like substances as auxins. The ultrafiltration allows to remove the excess amount of osmotic components and, therefore, to increase growth-stimulating activity of the preparations. Concentrates as a depot of HLS produced using micro- and ultrafiltration can meet the requirements of long-term liquid fertilizers. Permeates can be used in different dilutions as extraroot feeding because of mineral components and active fractions of humic-like substances.

Conflict of interest

The authors declare no conflict of interest.

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Author details

Sharipa Jorobekova¹ and Kamila Kydralieva^{2*}

1 Institute of Chemistry and Phytotechnologies, Bishkek, Kyrgyzstan

2 Moscow Aviation Institute (National Research Institute), Lomonosov Moscow State University, Moscow, Russia

*Address all correspondence to: kamila.kydralieva@gmail.com

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