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ABSTRACT

Soil fungi are actively involved in the processes of humic substances synthesis, transformation and mineralization due to production of extracellular nonspecific oxidative enzymes. The work was aimed to evaluate using spectral methods transformation dynamics for the humic product (HP) from lignosulfonate (HP_ligno) by filamentous soil fungal cultures Alternaria alternata and Trichoderma harzianum. Experiments showed that direct spectroscopic study of HP_ligno introduced into the nutrient medium and its transformation during fungal growth is challenging due to strong absorption of light by nutrient medium, development of absorbing fungal metabolites, partial utilization and destruction of HP by fungi and therefore due to the need to register tiny changes in overlapping bands. To accomplish that task we proposed a novel algorithm for processing the absorption spectra, which has not previously been used to study fungal cultures. We calculated the second-order derivative in respect to wavelength for absorption spectra measured during fungal growth and found characteristic “patterns” for introduced HP: a maximum at 270-285 and a minimum within 290-300 nm. The spectral index determined from amplitudes in the second-order derivative spectrum reflects the relative content of HP in the nutrient medium in presence of other absorbing components. We resume that two fungal strains utilized HP_ligno in the 0,02 and 0,1% concentrations better at 30 g/L sucrose than at 3 g/L in the medium. Thus the second-order differentiated absorption spectra helped to quantify degradation of the HP_ligno during fungal growth.

Keywords: Absorbance spectra, second derivative spectroscopy, optical indices, humic substances, fungal chromophores, Alternaria alternata, Trichoderma harzianum.

1. INTRODUCTION

The significance of humic substances (HS) in the environment is undeniable due to their participation in the global carbon cycle and soil structure [1], redox reactions [2], sorption and transport of pollutants, minerals and trace elements [3], maintenance of plant growth. Humic compounds are physiologically active substances regulating and enhancing metabolism in plants and soil. The HS play the most important role in the improvement of physical and chemical properties of soil, activation of the microflora, migration of nutrients. The HS global cycle is a combination of cycles of formation, transformation degradation, and mineralization. In general, HS possess a heterogeneous structure and are comprised mainly of aromatic, aliphatic, phenolic, quinoid and N-derived components covalently bound through C-C, C-O-C and N-C bonds [1]. The three-dimensional structure of HS has a great influence on their bioavailability and hence their biodegradability [4]. The accumulated experimental data reveal many aspects of the structure of HS but many remain debated, for example, the molecular mass of HS [5].

Two mechanisms of HS formation by microorganisms have been proposed. The first one suggests that microorganism activity and HS formation occur simultaneously; it assumes the existence of only macromolecular form of HS existence [6-8]. The second implies the possibility of the existence of both HS macromolecules and HS supramolecular compounds; it is believed that microorganisms are originally reproduced, which is followed by their death and HS formation [5,8].

In the nature HS (mainly humic acids and humin) are extremely resistant to biodegradation [4]. According to specialists, such stability is determined by several reasons; among them we mention two of the major importance: (1) Heterogeneity of structure: the spherical shape of their molecules, consisting of many heterogeneous units, irregularly connected by
heterogeneous bonds [9]. (2) The need to involve a system of extracellular enzymes, mainly non-specific oxidizing enzymes [10-12].

It is established that the soil filamentous fungi (micromycetes) are actively involved in the processes of HS synthesis, transformation and mineralization due to production of extracellular nonspecific oxidative enzymes [10-12]. However, the role of fungi in HS transformation is far from being well understood. Promising for research in this area are spectral methods. Spectral methods can be applied in a contactless or remote mode, which makes it possible to examine living organisms in vivo or in situ. Due to their high sensitivity, they allow monitoring even the tiny changes in the HS composition, which makes it possible to study in detail various aspects of mutual effects in the system "filamentous fungi – HS ". In recent years, spectroscopic studies have been used to study natural [13-16] and commercial HS [17-20]. Fourier transform infrared spectroscopy is used by the authors [21] to identify species of hyphomycetes and their mycotoxins. This method was used to study changes in the chemical structure of an organic substrate during its degradation by fungi [22].

The objective of this work was to evaluate transformation dynamics of the potassium humic product (HP) from lignosulfonate – lignohumate (further in the text – HP$_{\text{ligno}}$), as a representative of commercial HS preparation, by two soil fungal cultures Alternaria alternata and Trichoderma harzianum using measurements of absorption spectra and application of second-derivative spectroscopy to enhance spectral resolution for featureless spectral curves observed for HS and HP.

2. MATERIALS AND METHODS

2.1 Lignohumate preparation

Commercial humic preparation HP$_{\text{ligno}}$, lignohumate was used in this work as additional source of carbon for fungal cultures. It was produced by alkaline extraction from lignin-sulfonates or hydrolytic lignins (products of wood processing). This commercial product contains not only high-molecular fractions typical of many industrial HP analogues, but also a number of low-molecular humic components as well as a wide range of macro- and microelements. According to chemical analysis this HP contained 33.5 % C, and 0.25 %N, 3.64% H, 22.61% O, and 40% ash [23]. Aqueous solutions of HP$_{\text{ligno}}$ were prepared from distilled water.

2.2 Fungal cultures and experimental design

The objects of research were strains Alternaria alternata (Fr.) Keissl. and Trichoderma harzianum Rifai, which differ from each other by a number of growth and physiological characteristics. A. alternata is a dark pigmented species, for which synthesis of melanin was observed; the species is phytopathogenic. In contrast, T. harzianum is capable to control the development of pathogenic fungal cultures and, therefore, caused by them plant diseases.

Figure 1. Fungal colonies of Alternaria alternata (a) and Trichoderma harzianum (b) with the sporulation area on the Czapek agar medium with 30 g/L sucrose and 0.02% HP$_{\text{ligno}}$.

Growth of the fungal cultures was performed on the liquid Czapek medium with different contents of available carbon (sucrose) and different schemes of HP addition. HP$_{\text{ligno}}$ in biologically active concentrations of 0.02 and 0.1% was introduced into nutrient media with 3 or 30 g/L sucrose using two schemes: introduction of 0.02 and 0.1% HP$_{\text{ligno}}$.
simultaneously with micromycete inoculum and after their growth of fungal biomass for 7 days without HP in the nutrient medium. The sampling of supernatant liquid was performed on 7th and 14th days. Before spectral measurements the samples were filtered with 0.2 µm pore size filter representing cellulose acetate membrane (Chromafil, Macherey-Nagel, Germany).

2.3 Spectral measurements and optical indices calculation

Absorption measurements were carried out for liquid samples placed in quartz cuvette with 1 cm optical path with a double-beam spectrophotometer Unico (UK) within the spectral range 200-1000 nm and wavelength step 1 nm.

The first-order and second-order derivative absorption spectra were computed using differentiation of absorbance values with respect to wavelength. The first-order derivative manifests the wavelength intervals with higher spectral slopes in initial absorption spectra. The second-order derivative is useful to eliminate scattering background and resolve the overlapping individual components. The most characteristic feature of a second-order derivative is a negative peak with minimum at the same wavelength as the non-resolved peak hidden in the initial absorption band (primary absorption spectrum registered by spectrophotometer). Derivative spectroscopy previously helped to resolve spectral bands and discriminate scattering components in absorption spectra of commercial HP [24] and to characterize humic acids derived from commercially available HP of various sources [25].

3. EXPERIMENTAL RESULTS

3.1 Absorption spectra

Typical UV-vis absorption spectra for aquatic HS in natural water or HP aqueous solutions are without extremes showing absorbance values decreasing monotonically along with wavelength increase [24-26]. In the UV region some HP give weak absorption maxima around 230 and 285 nm, which can be resolved more distinctly as extrema in the second-order derivative spectrum [25]. Some shoulders at 230 and 285 nm can be observed for HP_ligno in water (see Figure 2). The reason for this is most probably the presence of low molecular weight phenolic compounds, released from source organic material (lignosulphonate) during its manufacturing process.

![Absorption spectra of HP_ligno in water and initial nutrient Czapeck medium (spectra reduced in 10 times) and supernatant liquid of Alternaria alternata cultures (AA) grown on different medium.](https://www.spiedigitallibrary.org/conference-proceedings-of-spie)

Figure 2. Absorption spectra of HP_ligno in water and initial nutrient Czapeck medium (spectra reduced in 10 times) and supernatant liquid of Alternaria alternata cultures (AA) grown on different medium.

As one can see from Figure 2 spectroscopic investigation of HP introduced into the nutrient medium and its transformation during fungal growth is challenging due to strong absorption of light by the nutrient medium, the
development of absorption bands of fungal metabolites, partial utilization and destruction of HP by fungi, and therefore due to the need to register tiny changes in weak overlapping bands in presence of strong scattering and medium absorption in the UV range.

To accomplish that task we proposed a novel algorithm for processing the spectral curves of absorbance values which has not previously been used to study fungal cultures.

3.2 Second-order differentiation of absorbance spectra

In this work we calculated second-order differentiated with respect to wavelength absorption spectra for the initial HP solution, nutrient medium and supernatant samples after 7 and 14 days of the fungal growth. Figure 3 shows second-order derivative spectra for supernatant liquid of *Alternaria alternata* cultures (AA) grown on different medium.

![Second-order derivative spectra](https://www.spiedigitallibrary.org/conference-proceedings-of-spie)

Figure 3. Second-order derivative spectra computed using differentiation of absorption spectra shown in Figure 2. “HP” – the range of maximal HP contribution, “FM” – the range of maximal contribution of fungal metabolites.

The analysis of differentiated absorption spectra let us to detect the following characteristic "patterns" which characterize introduced HP: in the UV region with maximum located at 270-285 and a minimum in the wavelength area of 290-300 nm. According to a decrease in the mentioned peaks amplitudes we concluded about the HP degradation during fungal growth. In this regard, to quantify the concentration of HP and its degradation we used the amplitudes in the second-order derivative spectrum at 284 nm (maximum contribution of HP) and 290 nm (minimum contribution of HP but significant contribution of fungal metabolites, FM). Thus we calculated the index \( I_{HS} = SD_{284} - SD_{290} \), which value reflects the relative content of HP, where \( SD_{284} \) and \( SD_{290} \) are amplitudes in the second-order derivative spectra taken at wavelength 284 and 290 nm correspondingly.

4. DISCUSSION OF RESULTS

Direct measurement of HP concentration in the nutrient medium during fungal growth is complicated by strong absorption resulting from fungal metabolites and growth medium, partial utilization and destruction of HP by fungi and therefore due to the need to register tiny changes in weak overlapping bands in the presence of other absorbing substances. However the analysis of the differentiated absorption spectra let us to calculate the index \( I_{HS} \), value reflecting...
the relative content of HP. Table 1 shows spectral index $I_{HS}$ reflecting the content of HP or humic-like substances in fungal cultures during their growth.

Table 1. Spectral index $I_{HS}$ in relation to the same value in the initial HP solution (%) reflecting the content of humic-like substances in fungal cultures during their growth.

<table>
<thead>
<tr>
<th>Filamentous fungi species</th>
<th>Growth duration</th>
<th>7 days</th>
<th>14 days</th>
<th>7 days</th>
<th>14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SPECTRAL INDEX</strong></td>
<td>$I_{HS}$ %</td>
<td>$I_{HS}$ %</td>
<td>$I_{HS}$ %</td>
<td>$I_{HS}$ %</td>
<td></td>
</tr>
<tr>
<td>Czapek medium supplemented with 30 g/L sucrose</td>
<td>without HP</td>
<td>17 ± 6</td>
<td>46 ± 11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>added after 7 days of fungal growth</td>
<td>-</td>
<td>78 ± 8</td>
<td>-</td>
<td>61 ± 6</td>
</tr>
<tr>
<td></td>
<td>added together with micromycete inoculum</td>
<td>89 ± 8</td>
<td>70 ± 10</td>
<td>52 ± 7</td>
<td>39 ± 6</td>
</tr>
<tr>
<td>Czapek medium supplemented with 3 g/L sucrose</td>
<td>without HP</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>added after 7 days of fungal growth</td>
<td>-</td>
<td>50 ± 9</td>
<td>-</td>
<td>60 ± 7</td>
</tr>
<tr>
<td></td>
<td>added together with micromycete inoculum</td>
<td>81 ± 6</td>
<td>88 ± 7</td>
<td>78 ± 6</td>
<td>49 ± 5</td>
</tr>
<tr>
<td></td>
<td>with 0.02% HP$_{ligno}$</td>
<td>-</td>
<td>87 ± 9</td>
<td>--</td>
<td>140 ± 20</td>
</tr>
</tbody>
</table>

The indices in the Table 1 were normalized to the values observed in the initial nutrient medium with HP. In most cases, the content of humic-like substances was decreasing during fungal growth; however, there were two cases where it exceeded the initial value in the medium, which means that some humic-like substances released by fungi resembled HP in optical properties.

Observing the $I_{HS}$ decrease in supernatant liquid during growth of fungal cultures, we can raise an issue of the HP$_{ligno}$ destruction by fungi depending directly on the growth duration and reversely on the HP$_{ligno}$ concentration (the higher is the initial HP content in the medium, the lower is the ability of fungi to its destruction). The analysis of the fluorescence spectra of supernatant liquid recorded during fungal growth supported the idea of the HP transformation by microscopic fungi.

5. CONCLUSIONS

Soil fungi are actively involved in the processes of humic substance synthesis, transformation and mineralization due to production of extracellular nonspecific oxidative enzymes. The objective of this work was to evaluate the transformation dynamics for the potassium humic product from lignosulfonate (HP$_{ligno}$) by filamentous soil fungal cultures *Alternaria alternata* and *Trichoderma harzianum* using spectral measurements.

Direct measurement of HS concentration in the nutrient medium and its transformation during fungal growth is challenging due to strong absorption of light by the nutrient medium, development of absorption bands of fungal metabolites, partial utilization and destruction of HP by fungi and therefore due to the need to register tiny changes in weak overlapping bands in the presence of strong scattering and medium absorption in the UV range. To accomplish that task we proposed a novel algorithm for processing the spectral curves of absorbance values which has not previously
been used to study fungal cultures. We calculated the second-order differentiated absorption spectra of the HP ligno during fungal growth and found characteristic "patterns" in the UV region which characterize introduced HP: a maximum located at 270-285 and a minimum in the wavelength area of 290-300 nm. The spectral index I_{HS}, determined from amplitudes in the second-order spectrum taken at 284 nm (maximum HP contribution) and 290 nm (minimum HP contribution), reflects the relative content of HP in the nutrient medium in presence of other absorbing components.

We resume that two strains Alteraria alternata and Trichoderma harzianum utilized HP ligno in the 0.02 and 0.1% concentrations better at 30 g/L sucrose than at 3 g/L in the medium. Effectiveness of the HP ligno consumption by filamentous fungi at lower sucrose concentration in the medium can be activated by their preliminary growth without HP addition which activates generation of fungal metabolites. Thus the second-order differentiated absorption spectra helped to quantify degradation of the HP during fungal growth.

6. ACKNOWLEDGEMENTS

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