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Biosorption and Biodegradation of Humic Substances by *Trichoderma viride*

Biosorpcija i biorazgradnja huminskih tvari s plijesni *Trichoderma viride*

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Summary

Mycelial pellets of ***Trichoderma viride*** *were used for biodegradation of naturally occurring and commercial humic substances from aqueous solutions. Experiments were carried out at 25 °C and pH = 6.0. Removal of humic substances from the aqueous phase was monitored by following the COD-elimination, fungal growth and decrease of absorbance at 370 nm. Within 10 days 42 % of HAS (soil humic acids), 44 % of FAS (soil fulvic acids) and 67 % of HAF (Fluka humic acids) were removed from the solution. It was found that beside degradation, adsorption of humic substances onto fungal mycelia play a significant role in this process.*

Sažetak

Micelijska zrnca ***Trichoderma viride*** *upotrijebljena su za razgradnju prirodnih i komercijalnih huminskih tvari u vodenoj otopini. Pokusi su provedeni pri 25 °C i pH = 6,0. Uklanjanje huminskih tvari iz vodene faze praćeno je mjerenjem KPK-vrijednosti, porasta biomase i smanjenja apsorbancije pri 370 nm. Tijekom 10 dana 42 % HAS (huminske kiseline izolirane iz tla), 44 % FAS (fulvinske kiseline izolirane iz tla) i 67 % HAF (Fluka huminske kiseline) uklonjeno je iz vodene otopine. Uočeno je da, pored razgradnje, adsorpcija huminskih tvari na micelijska zrnca zauzima značajno mjesto u tom procesu.*

Introduction

Humic substances are widespread on the earth's surface and represent the largest fraction of organic materials in aquatic, as well as terrestrial environments. They are formed when living matter, especially plants die and decay. Many of these substances are formed in soils, afterwards they find their way to lake, riverine and estaurine waters *(1)*. Other humic substances are formed directly within aquatic systems (especially ocean waters). Humic macromolecules are mainly built up by aliphatic carbon skeletons substituted with oxygen containing functional groups (carboxyl, alcoxy, hydroxy and carbonyl). This hetero-polycondensate is hardly degradable and therefore a very important carbon source in the natural carbon cycle *(2)*.

Being ubiquitous, humic substances are also present in drinking water causing some problems during the chlorination process. Trichalomethanes (THMs), which came under suspicion of being cancerogenic, are formed when chlorine reacts with humic substances in raw water to disinfect it for municipal drinking water supplies *(3)*.

Furthermore, humic substances can mobilize heavy metals *(4)* and persistent organics such as pesticides (PCBs, DDT) and polynuclear aromatic hydrocarbons *(5)* and thus increase the environmental distribution of inorganic and organic micropollutants.

Accordingly, removal of humic substances is now one of the important objectives in water purification technology. There are many references concerning the use of different microorganisms for the degradation of humic substances in water, such as aquatic microbial communities *(6,7)*, pure bacterial culture of *Pseudomonas (8)*, of actinomycetes *Streptomyces viridosporus* and *Streptomyces* sp. *(9)* and several fungi including *Phanerochaete chrysosporium (9,10)*, *Rhizopus arrhizus (11)* and *Aspergillus glaucus (12,13)*.

The aim of this work was to study the biosorption and biodegradation of humic and fulvic acids in an aqueous solution using mycelial pellets of *Trichoderma viride*.

Materials and Methods

Isolation of humic and fulvic acids

Humic substances used in this study were obtained from a commercial source (Fluka, humic acids, designed as HAF) and were also isolated from the uncultivated soil obtained from a wooded hill Medvednica. Isolation of humic (designed as HAS) and fulvic (FAS) acids from the soil was carried out, with a few modifications, according to the method described by Malcolm *(14)*. Complete procedure is shown schematically in Fig. 1.

Microorganism and Cultivation Conditions

The mold used in these experiments was *Trichoderma viride* (from the Faculty of Chemical Engineering and Technology Culture collection, Zagreb). This strain was routinely maintained and checked for purity on malt agar slant at 28 °C.

Precultures were prepared in 500 mL conical flasks containing 100 mL of potato dextrose (PD) medium *(15)* on a rotary shaker (200 rpm) »Gallenkamp« IH-460 at 25 °C and pH = 6.7 for 3 days. Inoculation was carried out by spore suspension at a final density of 3.0×10^6 spores/mL.

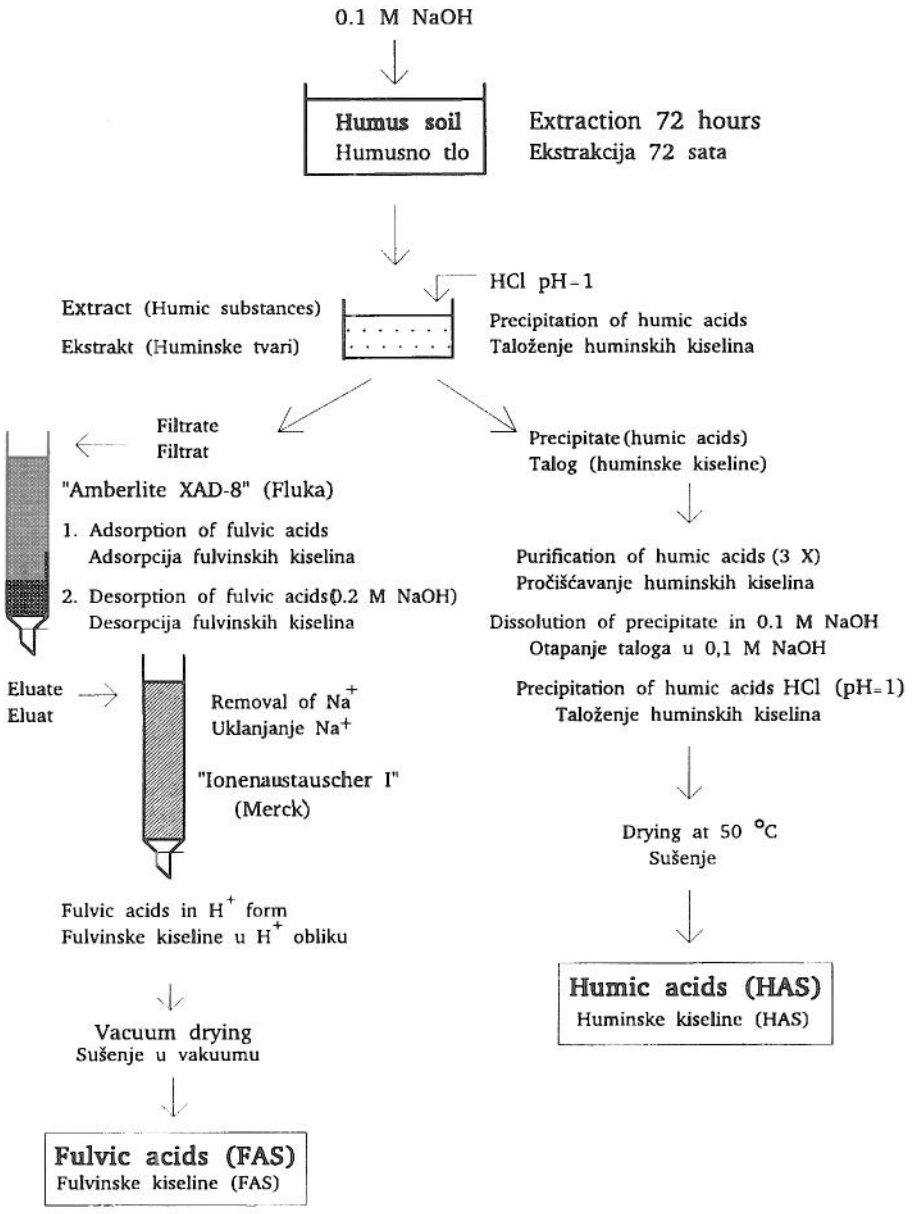

Fig. 1. Scheme of the separation of humic and fulvic acids from soil
Slika 1. Shematski prikaz odjeljivanja huminskih i fulvinskih kiselina iz tla

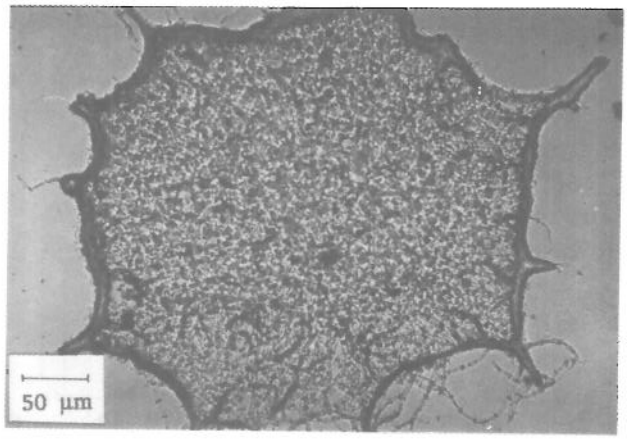

Fig. 2. Photomicrograph of the cross section of mycelial pellet of *T.viride* grown in PD medium

Slika 2. Fotografski snimak presjeka micelijskog zrnca *T.viride* uzgojenog u PD podlozi

The mycelium pellets that developed were then harvested over a nylon filter (20 mesh), washed several times with distilled water, slightly squeezed to remove excess liquid and transferred as desired.

For biosorption and biodegradation experiments the following basal medium was prepared: 0.2 g NH_4NO_3, 1.0 g KH_2PO_4, 0.5 g $MgSO_4$, 0.5 g KCl, 0.5 g peptone for 1 L culture. This medium was supplemented with 0.002 g/L of thiamine. Humic acids were sterilized by filtration (pore size 0.45 µm) and incorporated into the previously autoclaved basal medium.

Experimental Procedures

Biosorption experiments were carried out at 25 °C and pH = 6.0, during 24 hours, on a rotary shaker at 200 rpm. The standard charge of mycelial pellets was 1.0 g (fresh weight) or 0.051 g (dry weight). Reaction volumes were 100 mL of basal medium containing humic substances in the concentration ranges as follows: HAS 25-95 mg/L, FAS 35--115 mg/L, HAF 15-55 mg/L. Concentration of humic substances was determined spectrophotometrically (λ = 370 nm) using a Specol 210 spectrophotometer (Zeiss-Iskra MA 9325). In all experiments duplicate runs were made.

Biodegradability of HAS, FAS and HAF in an aqueous solution by mycelial pellets of *Trichoderma viride* was monitored by following the decrease in absorbance at λ = 370 nm and decrease of COD-value at 25 °C and pH = 6.0 during 10 days. Data were converted to a concentration of humic substances (mg/L) using calibration curves. Growth of mold was assessed by the dry weight as determined after collection of the mycelia and drying at 105 °C on weighted filters. All experiments were carried out in duplicate in 100 mL of basal medium containing 1.0 g of mycelia (fresh weight) and two concentrations of HAS (40 and 80 mg/L), FAS (42.5 and 85.0 mg/L) and HAF (25 and 50 mg/L) respectively.

Table 1. K, $1/n$ and regression coefficients of the Freundlich adsorption isotherms

Tablica 1. K, $1/n$ i koeficijenti regresije Freundlichovih adsorpcijskih izotermi

Humic substances Huminske tvari	$1/n$	$K \cdot 10^6$	r^2
HAS	0.64	57.6	0.99
FAS	0.67	47.5	0.97
HAF	0.73	96.8	0.97

Results and Discussion

The rate of biodegradation of humic substances in an aqueous solution may be affected by a variety of factors, including adaptation of microorganisms and conditions under which experiments were undertaken. Adaptation may be a result of several alterations in the structure and function of microbial cells including induction or derepression of enzymes, genetic change and growth of specific degrading organisms *(16)*. However, literature data indicate that removal of humic substances from aqueous phase was not only the consequence of their biodegradation, but it was partly caused by adsorption of humic macromolecules on mycelial pellets.

The first experiment in this study was undertaken to determine the rate of biosorption in the process of humic substances removal by mycelial pellets of *T. viride*. Photomicrograph of mycelial pellet is shown in Fig. 2. Experimental results showed that equilibrium adsorption was established within 5 hours. The adsorption curves of HAS, FAS and HAF over the range of concentrations studied were analogous to the Freundlich isotherm:

$$q = K(\gamma_e/\mathrm{mgL}^{-1})^{1/n}$$

where q is the amount of humic substances adsorbed (g/g of mycelia (fresh weight)), γ_e is the equilibrium concentration of humic substances (mg/L) and K and n are adsorption coefficients. Freundlich K and $1/n$ values for the adsorption of HAS, FAS and HAF (Table I) were obtained from the intercepts and the slopes of the logarithmic adsorption plots shown in Fig. 3. The highest adsorption coefficient *(K)* obtained was related to the adsorption of commercial sample of humic acids (HAF),

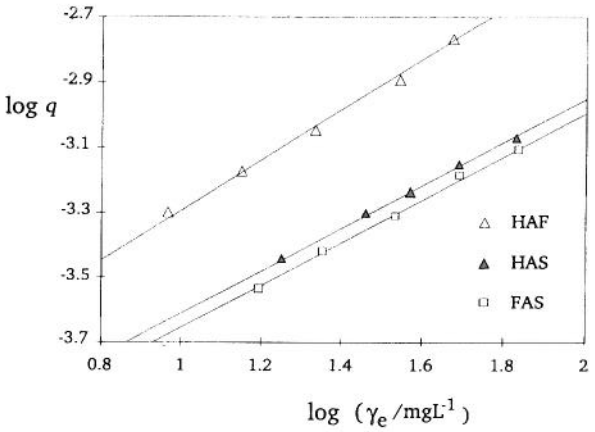

Fig. 3. Freundlich adsorption isotherms of HAS, FAS and HAF

Slika 3. Freundlichove adsorpcijske izoterme HAS, FAS i HAF

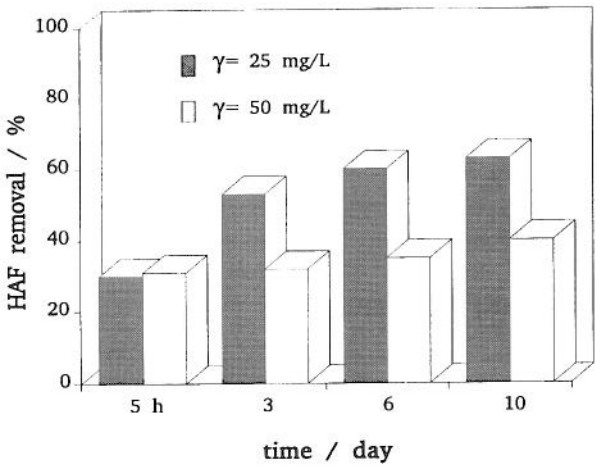

Fig. 4. Removal of HAF from the aqueous solution by *T.viride*
Slika 4. Uklanjanje HAF iz vodene otopine s *T.viride*

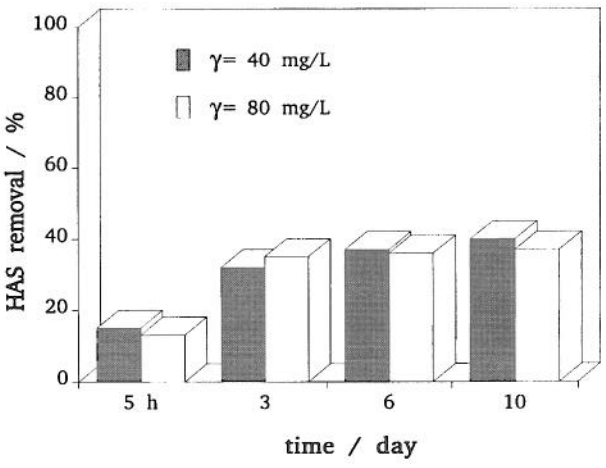

Fig. 6. Removal of HAS from the aqueous solution by *T.viride*
Slika 6. Uklanjanje HAS iz vodene otopine s *T.viride*

while adsorption coefficients of natural soil humic and fulvic acids were significantly lower.

After that initial fast sorption the aqueous concentration of humic substances remained unchanged for at least 24 hours. Afterwards, significant decreasing of humic substances concentration continued through a 10-days incubation period during which COD-elimination and mycelial growth were also observed. Figures 4, 5 and 6 show the removal of humic substances present at two concentration levels. First values (t = 5 hours) correspond to the amount of humic substances bound to the mycelial pellets by adsorption. Further removal appeared to be a result of degradation activity of the fungus and it started after three days. HAF and FAS concentrations during 10 days of incubation decreased yielding quite similar patterns. Better removal of these substances was obtained at low concentration level, although total amount of HAF removed from the aqueous phase was much higher. In the experiments with test solutions containing HAS, percent of humic substances removed was not dependent upon concentration.

These observations were confirmed by the measurements of COD-elimination in the solutions containing low concentration of humic substances. COD-value in the aqueous solution containing HAS was decreased by 46.4 %, 42.2 % when FAS was present and 61.8 % when commercial sample of humic substances (HAF) was studied. By weighting the mold biomass before and at the end of experiment, it was found that the amount of biomass in all cases was approximately doubled from 0.48 g/L to 1.10 g/L (dry weight). When the same amount of *T.viride* conidia were directly inoculated and incubated in culture medium (25 °C and pH = 6.0) with 25.0 mg/L of HAF, mycelial growth and decolorization of test solution

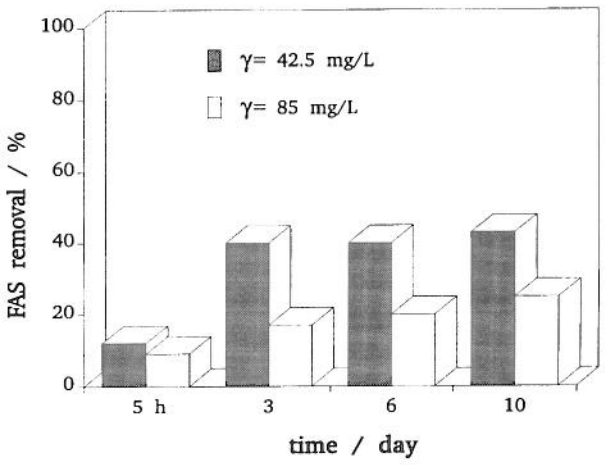

Fig. 5. Removal of FAS from the aqueous solution by *T.viride*
Slika 5. Uklanjanje FAS iz vodene otopine s *T.viride*

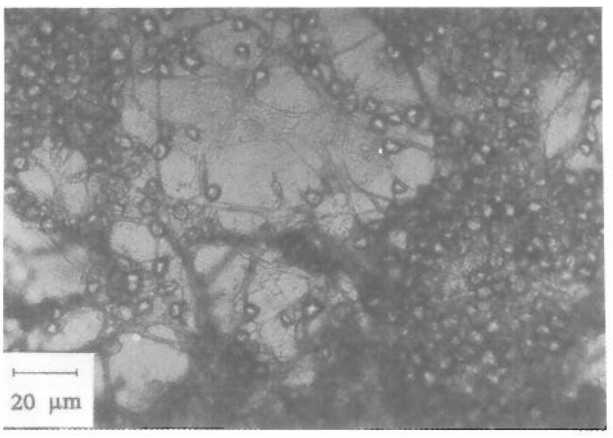

Fig. 7. Photomicrograph of mycelial pellet of *T.viride* after 10 days of growth in medium supplemented with HAF at inoculation
Slika 7. Mikroskopska slika micelija *T.viride* nakon 10-dnevnog uzgoja u podlozi s dodatkom HAF pri nacjepljivanju

started after an initial lag of approximately 3 days and continued through the 10 days of experiment. Mycelial pellets were smaller (0.1-0.3 mm in diameter) and not as compact as the ones formed in PD medium, but the total removal of HAF was quite high (45 %) considering the fact that this removal was primarily the result of biodegradation, while adsorption was negligible. During the incubation period 0.77 g/L of biomass (dry weight) was produced. It is supposed that humic substances influenced the morphology of mold, inducing the formation of swollen cells and frequent branching of growing hyphae (Fig. 7). Leštan and co-workers *(17)* reported that in the swollen cells of *Phanerochaete chrysosporium* the specific ligninolytic activity was increased, which may imply that in the swollen cells of *T.viride* increased enzymatic activity occurred as well.

All the results obtained suggest that, even though adsorption of humic substances onto mycelial pellets plays a significant role in the process of humic substances removal, *Trichoderma viride*, as a biological catalyst, possesses an enzymatic system which is, at least partially, responsible for the degradation and therefore for the removal of humic macromolecules from the aqueous solution. Concerning the fact that values obtained for HAF (Fluka-commercial sample of humic acid) are considered high comparable with values obtained by measuring adsorption coefficients and biodegradation rates of natural humic (HAS) and fulvic acids (FAS) it is evident that a representative sample of natural humic substances, isolated from the local environment, is an obligatory prerequisite.

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